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Collective Memory in Leafcutter Ants

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Collective Memory in Leafcutter Ants

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Abstract

Collective Memory in Leafcutter Ants

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Like individual organisms, collective organisms such as slime molds and social insect colonies, go through different life-history stages. Collective organisms also show cognitive properties, such as decision-making, learning, and memory. Here, I study the earliest phase of collective life in *Atta texana* leafcutter ants (Chapter 1) and investigate the duration and mechanisms of collective memory of leaf rejection in *Atta colombica* (Chapter 2 & 3). In Chapter 1, I measured a mortality rate of 83.7% in foundresses from the time of the mating flight to the emergence of the first workers. And, I found high variation in early growth trajectories of colonies in terms of worker number and fungal garden volume. I found two species of entomopathogenic fungi that were likely causes of mortality in *Atta texana* foundress queens. In Chapter 2, I examined the mechanisms that allow the maintenance and decay of a learned behavior within leafcutter ant colonies. Specifically, I studied the effects of 1) worker-worker interactions, 2) differential information use by certain workers (old, experienced workers vs young, naïve workers), and 3) worker longevity on the maintenance and decay of collective leaf rejection. In Chapter 3, I studied how individual social learning strategies (e.g., 'who to copy' and 'when to copy') contribute to the maintenance of colony-level memory. To answer this question, I manipulated colony compositions and measured which groups of foragers copied the foraging

preferences of their nestmates based on their own age and past experiences, and on the age, past experiences, and task group (i.e., body size) of their nestmates. I found that leafcutter ants use multiple positive feedback mechanisms of information transfer and a state-dependent social learning strategy, to allow leaf rejection behavior to spread through colonies. When these behavioral rules exist in the context of the constant, gradual turnover of individuals, and other age-based differences in behavior, the emerging whole-colony system is both flexible and capable of retaining information.

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Introduction

Collective behavior emerges from individuals responding to their local environment without an omniscient director to coordinate them (Sumpter 2006). Navigation in flocks of birds (Berdahl et al. 2018), consensus decision making in cockroaches (Ame et al. 2004), and efficient division of labor in social insects (Seeley 1995) function because individuals follow simple rules based on their perception of social and environmental cues. Research in collective animal behavior has had huge interdisciplinary impacts (Dorigo et al. 2000; Marshall and Franks 2009; Rubenstein et al. 2014). However, models of collective behavior often ignore two key aspects 1) how collective behavior changes over time, and 2) how the turnover of individuals within a group contributes to collective patterns, as the most knowledgeable individuals die and are replaced by newly born, naïve individuals.

Collective memory exemplifies this change over time in collective behavior. Individual memory is often defined as the encoding, storage, and maintenance of information by individuals. I define collective memory as the encoding, storage, and maintenance of information by collectives. The key diagnostic for learning or memory in animal behavior is to compare two groups, one that has had a particular experience and one that has not (Shettleworth 2010). This definition of learning is focused on outcomes, rather than on mechanisms. In many cases it may be important to make a mechanistic distinction between learning and other forms of behavioral plasticity. However, from an evolutionary or ecological perspective, many different mechanisms could be acted upon to produce the similar outcomes. When making comparisons across levels of biological organization, a dual focus on similar outcomes and their possibly diverse mechanisms can lead to fruitful insights across disciplines.

Individuals within groups accumulate experience over their lifetimes, both from their social partners and from their broader environment. Individual learning, social learning, and individual turnover likely have profound effects on collective behavior over long timescales. Group-level phenomena of long-term memory suggests that interactions between groupmates, both directly and mediated through their environment, may serve as the storage, maintenance, and retrieval of collective memories. For example, seed-harvesting ants learn individually which seeds they should harvest and how to harvest particular types of seeds efficiently. Colonies of seed-harvesters behave as if they are familiar with particular seeds up to 180 days. Though untested, it is likely that collective memory in this system could be mediated by underground seed caches, and thus is "stored" in these caches (Johnson 1991; Johnson et al. 1994). Slime molds display habituation learning when they encounter new chemicals. They are able to transfer these habituated memories when fused with other slime molds (Vogel and Dussutour 2016). And, they are able to remember their habituation even after a one-month dormancy period (Boussard et al. 2019). Ungulate migration patterns appear to be learned, modified and remembered over the course of generations. After a translocation of several ungulate herds, these herds initially do not migrate. However, over multiple decades, herds increase their knowledge of resource seasonality and increase in the propensity to migrate (Jesmer et al. 2018). In these systems individual learning and memory is enabled or enhanced through social interactions to maintain memory storage in the group.

Social insects offer especially interesting opportunities for the study of collective memory. First, due to inclusive fitness, they may have evolved mechanisms of nestmate interaction to promote memory maintenance. Second, often the lifespan of colonies is much longer than that of workers (Giraldo and Traniello 2014), and in such cases it is possible that associations could be

transferred from one worker cohort to the next such that they outlive individual workers. Lastly, social insect colonies have been heralded as great study systems for collective cognition generally (Seeley 1995; Couzin 2009; Razin et al. 2013).

The mechanisms by which collective memory is formed, maintained, and forgotten are particularly interesting as they may give clues as to how behavioral plasticity can be maintained at any level of organization. In animal brains, memory is stored in the connections between neurons. In animal groups, memory could be stored in individual brains and shared via social learning (Whiten et al. 1999; Jesmer et al. 2018). These memories could also be stored in the environment as in physical and chemical foraging trails in both insects (Czaczkes et al. 2015) and slime molds (Smith-Ferguson et al. 2017), in food stores (Johnson 1991; Johnson et al. 1994; Dornhaus and Chittka 2005), or in built structures (Grasse 1959).

I studied the colony-level outcomes, and individual-level mechanisms of collective memory of learned leaf rejection in leafcutter ants. Specifically, I asked , are leafcutter ant colonies able to transmit the knowledge of a learned behavior from old, experienced ants to newly born, naïve ants? And if so, what mechanisms allow leafcutter ant colonies to retain information while also remaining behaviorally flexible to changes in the environment?

Leafcutter ants face the difficult task of deciding which leaves to use as substrate to grow gardens of fungus in underground chambers (Weber 1972). The ants and their fungus are obligate mutualists; the fungus metabolizes leaf material provided by the ants, and the ants eat the mycelium of the fungus. Tropical leafcutter ants collect leaves from as many as 50-80% of the plants in their foraging range (Cherrett 1989) and bring them to their fungus gardens, which provide feedback to the ants on the quality of the leaves they have collected (Herz et al. 2008). The quality of plants harvested by leafcutter ant colonies changes over time. Certain leaves are acceptable only for

certain parts of the year (Rockwood 1976). Individual workers live around 6 months at most (Fowler et al. 1986); resources that are seasonally acceptable may therefore be completely novel to workers when seasons change.

This dissertation focuses on collective memory of learned leaf rejection. Laboratory and field studies have shown that leafcutter ants learn to reject species of plants whose leaves are detrimental to fungus garden growth (Herz et al. 2008; Saverschek et al. 2010a). Rejection is based on cues from their fungus gardens that are produced for the 2-3 days after harmful leaves have been incorporated into a garden (Herz et al. 2008). After this cue from the fungus garden has decayed (after Day 3), there are numerous other ways that foragers learn both to avoid a particular type of leaf and to prefer other types of leaves. Ants learn leaf avoidance and preference from bits of rejected leaf material in the waste (Arenas and Roces 2018), from beneficial leaf material in the fungus chamber (Arenas and Roces 2018), and from brief interactions with leaf fragments carried by workers on the foraging trail (Roces 1990, 1994; Farji-Brener et al. 2010).

After learning to reject a plant species, leafcutter ant colonies have been shown to continue to avoid this species of plant for as long as 30 weeks in the laboratory (Ridley et al. 1996) and for 12-18 weeks in the field (Saverschek et al. 2010a). Leaf rejection persists for many weeks without any further reinforcement that the previously treated leaf type is still harmful, and indeed it is no longer harmful. It is likely that new workers emerge and mature to foraging age during this time, and these workers are naïve to which leaves had been treated, i.e., they would not have themselves experienced cues from the fungus designating the previously treated leaf type as harmful.

My dissertation aims to address how 1) life history, i.e., age-based changes in cognition, 2) worker turnover, and 3) social learning contribute to a collective memory system that is able to

maintain the memories of a minority of experienced individuals while remaining flexible in the face of changing environmental conditions.

In Chapter 1, I studied the earliest phase of an *Atta* leafcutter colony's life. I found three likely causes of mortality in *Atta texana* foundress queens, quantified the foundress mortality rate from the time of the mating flight to the emergence of the first workers, and quantified early growth trajectories of colonies in terms of worker number and fungal garden volume.

In Chapter 2, I examined the mechanisms that allow the maintenance and decay of a learned behavior within leafcutter ant colonies. Using ant colonies as a model for collective cognition enables the direct manipulation of individuals whose interactions preserve a memory within a group in a way that remains challenging for the direct manipulation of neurons in a brain. Specifically, I studied the effects of 1) worker interactions, 2) differential information use by certain workers, and 3) worker longevity on the maintenance and decay of collective leaf rejection.

In Chapter 3, I studied how individual social learning strategies, i.e., 'who to copy' and 'when to copy', contribute to colony-level memory maintenance. To answer this question, I manipulated colony compositions and measured which groups of foragers copied from their nestmates based on their own age and past experiences, and the age, past experiences, and task group (i.e., body size) of their nestmates.

My dissertation is one of few studies to determine how individual-level mechanisms and individual turnover lead to long-term patterns in collective cognition. I found that leafcutter ants use multiple positive feedback mechanisms of information transfer and a state-dependent social learning strategy, to allow leaf rejection behavior to spread through a colony. When this behavioral rule exists in the context of the constant, gradual turnover of individuals, and other age-based differences in behavior, the emerging system is both flexible and capable of retaining information.

Chapter 1: Foundress queen mortality and early colony growth of the leafcutter ant, *Atta texana* (Formicidae, Hymenoptera)[1]

ABSTRACT

Nest-founding queens of social insects typically experience high mortality rates. Mortality is particularly severe in leafcutter ants of the fungus-growing ant genus *Atta* that face the challenge of cultivating a delicate fungus garden in addition to raising brood. We quantified foundress queen survivorship of *Atta texana* that were collected in northwest Texas and maintained in single-queen laboratory nests, and we track[1]ed the rate of colony growth during the first precarious months of the colony lifecycle. Ninety days post-mating flight, only 16.3 % of 141 of the original queens had survived, and colony growth rates varied markedly across the surviving colonies. Worker production was weakly correlated with fungus garden growth over the course of early colony development. Dead queens became overgrown by the parasitic fungi *Fusarium oxysporum* (26 % of dead queens) and *Aspergillus flavus* (34 %), and these fungi are therefore possible causes of queen mortality. The phorid fly *Megaselia scalaris* emerged from one dead queen, but was unlikely the cause of death. Under natural conditions, intense competition between conspecific colonies can amplify small differences in initial growth rates to generate drastic differences in colony fitness. The observed variation in colony growth rate therefore suggests that colony growth is likely an important target for selection to maximize fitness in *Atta texana*.

[1] This chapter is published as: Marti H, Carlson A, Brown B, Mueller U (2015) Foundress queen mortality and early colony growth of the leafcutter ant *Atta texana* (Formicidae, Hymenoptera). Insectes Soc 62:357–363. For this study I designed the objectives and methods with UGM, collected data on colony growth and survivorship, analyzed the data, made figures, and wrote the first draft of the manuscript. AC sequenced and identified fungal pathogens and BB identified *Megaselia scalaris.*

INTRODUCTION

The nest-founding stage is a particularly critical stage in the life history of social insects (Oster and Wilson 1978). Nest-founding queens typically experience low survivorship, which creates a selective bottleneck where a very small proportion of surviving queens contribute to the next generation (Brian 1965; Wilson 1971; Cole 2009). Direct observations of foundress survivorship are lacking for most ant species, but for those studies that do exist (e.g., *Pogonomyrmex occidentalis*, *Crematogaster ashmeadi*, *Solenopsis invicta*, *Atta bisphaerica*), the reported percentages of queens surviving to produce incipient colonies range between 0.09 and 7.6 % (Cole 2009 and references therein). Using the Texas leafcutter ant, *Atta texana*, we expand on earlier work by combining new observations on the causes of queen mortality and early colony growth dynamics with previously published information to elucidate trends common across leafcutter ant species (*Atta* and *Acromyrmex*).

Leafcutter ants of the genus *Atta* have some of the lowest estimates of foundress survivorship among ants (Jacoby 1944; Autuori 1950; Fowler 1987; Cole 2009). Direct observation of foundress survivorship in *Atta bisphaerica* estimated only 0.09 % of queens surviving the nest-founding stage (Fowler 1987). Low survivorship in *Atta* and other fungus-growing ants is thought to be due to the compounded challenges of cultivating a delicate fungus garden while raising the first worker-brood, avoiding predators, resisting execution by conspecifics, and coping with pathogens and parasites (Weber 1972; Fowler et al. 1984, 1986; Fowler 1987).

Of colonies that do survive the nest-founding stage in claustral *Atta spp.* in the field, the first workers open the sealed foundress-nest approximately 1.5–3 months after founding (Huber 1905; Autuori 1942; Moser 1967; Weber 1972). In the laboratory, measures of foundress survivorship exist for *A. texana*, including comparison of survivorship in multiple-queen versus

single-queen nests (Mintzer and Vinson 1985; Mintzer 1987). Survivorship is higher in the lab than the field; and fitness is higher for multiple-queen than single-queen nests, as measured by survivorship, worker number and fungus garden size (Mintzer and Vinson 1985; Mintzer 1987). Nests founded by multiple queens occur at a low rate across the range of *A. texana*, 13 % for a population in central Texas (Mintzer and Vinson 1985), and 2.5 % nests in the population studied here at the range limit in northwest Texas (Mueller unpublished observation).

While colony growth is typically measured by the number of workers, the size of the fungus garden is also a key component of colony growth for attine ants, because stored energy resources of a founding queen are allocated to both the production of workers and the cultivation of the incipient fungus garden (Cahan and Julian 1999; Seal and Tschinkel 2007; Clark and Fewell 2014). Previous studies on the semi-claustral, attine species *Acromyrmex versicolor* (Cahan and Julian 1999; Clark and Fewell 2014) and *Trachymyrmex septentrionalis* (Seal and Tschinkel 2007) suggest that the growth relationship between fungus garden size and worker number is variable over the first few months of colony growth and stabilizes sometime before colonies reach sexual maturity. The relationship between the early growth rates of worker number and garden size are unknown for *Atta*. To explore the early growth dynamics in *Atta texana* colonies, we quantified foundress queen survivorship, tracked the rate of colony growth of surviving colonies during the first 3 months after nest founding, and compared growth rates of worker number and fungus garden size.

The causes of death for *A. texana* queens are not well known. Past studies of other ant species suggested that exposure to parasites and pathogens during the mating flight could be significant causes of death (Fernandez-Marin et al. 2004; Augustin et al. 2011). Furthermore, without workers to perform social immune behaviors, such as allogrooming, solitary founding

queens are especially susceptible to infection (Ho and Frederickson 2014). *Escovopsis spp.* are the most commonly studied fungal parasites of gardens, which can infect the gardens of many tropical fungus-growing ants (Currie et al. 1999; Meirelles et al. 2015). *Escovopsis* was not found to infect gardens of *A. texana* in central Texas (Rodrigues et al. 2011a), and has only been found so far in a few *A. texana* gardens in south Texas (Mueller, unpublished observation). Other parasitic fungi are more frequently found in *A. texana* gardens, including *Syncephalastrum racemosum*, *Fusarium oxysporum*, *Aspergillus flavus*, and *Acremonium polychromum* (Rodrigues et al. 2011a; Seal and Mueller 2014). Ecologically similar fungi have been isolated on newly mated *Atta capiguara* and *Atta laevigata* queens in Brazil, such as *Acremonium spp.*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium graminearum*, *Paecilomyces lilacinus*, *Trichoderma atroviride*, and *Beauveria bassiana* (Rodrigues et al. 2011b).

Phorid fly parasitoids of several genera are also a source of mortality in leafcutter ants, including *Myrmosicarius* (Borgmeier 1928), *Procliniella* and *Stenoneurellys* (Borgmeier 1931), *Apocephalus* (Brown 1997), *Eibesfeldtphora* and *Lucianaphora* (Disney et al. 2008), and Neodohrniphora (Disney et al. 2009). These parasitoids lay their eggs in the bodies of ant-hosts, which then pupate in and emerge from the ants' bodies (Porter et al. 1995; Brown 1997). During our study of mortality of incipient *A. texana* colonies, we examined two types of parasitic fungi and a phorid fly as possible causes of death of *A. texana* queens.

METHODS

Ant collection and rearing

Atta texana queens searching for nest sites or actively digging nests were collected from three sites in northwest Texas within 2 h following their mating flights. Queens were collected on

May 25th 2014 in Glen Rose, TX (N32.24899 W97.73760, elev. 194 m) between 6:30 and 8:00 am (n = 14 queens); and on May 26th 2014 in Newcastle, TX (N33.19405 W98.73891, elev. 351 m) between 6:00 and 7:00 am (n = 60) and in Fort Belknap, TX (N33.15118 W98.74026, elev. 358 m) between 7:00 and 8:00 am (n = 67). Newcastle and Fort Belknap mark the northwestern range limit of *A. texana* (Mueller et al. 2011a, b). Mating flights occurred during early dawn on days following heavy rainfall (e.g., alate reproductives departed 5:44–5:56 am from a nest at Fort Belknap). Within 15–30 min after departure from their nest, the first queens (presumably having mated) were attracted to bright streetlights, where they were collected and transported to the University of Texas at Austin.

Queens were collected into sterile vials in the field, and within 4 h queens were transferred into two types of cylindrical containers, large containers (4 cm 9 5.5 cm diameter; n = 78) and small containers (5.5 cm 9 2 cm diameter; n = 63), which were filled with moistened dental plaster to within 1–1.5 cm of the top. The forceps used to transfer queens from collection vials to plaster containers were sterilized for each queen. To reduce the introduction or cross-contamination of microbes, all containers had tight lids that permitted limited gas exchange.

Queens were kept at 22–24 C in a room without specific light or humidity regulation; however, humidity in nest containers was always near 100 % due to the moistened plaster. Queens were checked for mortality 9, 12, 45, 70, and 90 days following collection. Queens that did not initiate a garden or lost their fungus gardens were scored as functionally dead, as garden-less queens are unlikely to survive in the field.

Once workers began to pupate (about 45 days after the mating flight), colonies were moved to nest boxes with two 7.5 9 7.5 cm plastic chambers connected by rubber tubing (Mehdiabadi et al. 2006). Ants and fungus gardens were transferred with ethanol-sterilized forceps. The bottom of

one of the two connected chambers was lined with moistened dental plaster to generate a humidified nesting chamber, while the other chamber was left empty as a relatively dry foraging chamber. Plaster chambers were kept covered after pouring the plaster to reduce the amount of introduced microbes. The plaster was re-moistened once every week with deionized water. Once a colony had at least 20 workers (about 20 days after emergence of the first worker and about 70–75 days after nest initiation), each colony was fed twice weekly with approximately 100 mg of minced orange pith.

To estimate colony growth rates, we counted the number of workers in each colony and measured the volume of each fungus garden. Because workers were moving, we counted the workers three times and calculated the average of these counts. If the range of the first three counts was greater than seven workers, we counted an additional three times and averaged across all six counts. Additional counts were typically needed when colony size exceeded 60–70 workers. Workers were counted twice per week during the month of July (50–66 days post-mating flight) and once per week for the month of August (70–90 days post-mating flight).

To track the growth of fungus gardens, we measured the volume of each garden once per week beginning on August 4th (70 days post-mating flight), using a method modified from Seal et al. (2014). We estimated the volume of each garden by overlaying a 5-mm grid on the top of each nest box and counting how many grid squares were filled more than 50 % with fungus garden. We also took a measurement of the maximum height of the garden using the same grid squares against the side of each nest box, but measuring the height to the nearest of a grid square. Because the height of the gardens was approximately uniform, the grid-square estimate and height were multiplied to estimate total garden volume. It was not possible to measure fungus garden volume blindly without awareness of the approximate worker number, because no second naïve

experimenter was available. Using the program R 3.1.1 (R Core Team 2013), we tested the relationship between fungus garden volume and worker number at days 70 and 90 using linear regressions.

Fungal Isolation and DNA Sequencing

Of 141 queens collected, 50 died within the first 2 weeks and were discarded without scoring possible causes of mortality, but we noticed that many of these dead queens had become overgrown with fungi, so the remaining 91 queens were carefully monitored for the emergence of parasitic fungi. For each fungal morphotype visible on these queens, we chose ten queens with the greatest spore and mycelium coverage for isolation. Isolations were performed July 11th, 7 weeks after the initial collection date. Fungal tissue or spores from the lower bodies of dead specimens were streaked on potato-dextrose agar (PDA) plates (DIFCO; Becton, Dickinson and Company; Sparks, MD 21152 USA). Fungi grew on PDA for 1 week at room temperature, at which point the fastest-growing strain of each morphotype was chosen for sequencing. A 1–2 mm piece of tissue was removed with a sterile scalpel and transferred to a 10 % Chelex solution, and DNA was extracted using a basic Chelex protocol (Sigma-Aldrich, St. Louis, MO 63103 USA). The intergenic-spacer (ITS) region of rDNA was amplified for two, morphologically distinct samples using the primer pair ITS4/ITS5, then sequenced on an ABI 3100 Automated Sequencer (Applied Biosystems), following the methods of Rodrigues et al. (2011b). Raw sequence information was edited and assembled in Geneious v 6.0.3. Sequences are deposited at NCBI Genbank under accessions KM284800 and KM284801.

RESULTS AND DISCUSSION

Colony survivorship

By August 24th, 90 days post-mating flight, 23 of the 141 original queens had survived, for a survivorship of 16.3 % (Fig. 1.1). When examined by collection site, the percentages of queens surviving after the first 3 months were 0 % (Glen Rose, TX; n = 14), 16.7 % (Newcastle TX; n = 60), and 19.4 % (Fort Belknap TX; n = 67). The highest mortality rates occurred within the first 9 days after the mating flight, and survivorship did not stabilize until the third month of colony establishment (Fig. 1.1). Survivorship rates observed here in the lab are higher than those reported from field studies for other *Atta* species (0.09–6.6 %; Jacoby 1944; Autuori 1950; Fowler et al. 1986; Cole 2009). Unlike our lab colonies, *Atta spp.* nests in the field are presumably exposed to additional pressures such as predation, execution by neighboring *Atta spp.* nests, desiccation of fungus gardens, and diseases present in natural soil.

Colony Growth

The first workers began to eclose on days 50–55 after nest initiation, which was somewhat later than previously reported for this species. Mintzer and Vinson (1985) and Mintzer (1987) reported worker eclosure after 36–40 days, and 32–42 days, respectively, for laboratory colonies. In field colonies, Moser (1967) estimated first worker eclosure at 40–50 days. It is likely that brood developed slower in our colonies because they were kept at lower temperatures (22–24 C) compared to those of Mintzer (1987; 27 C).

Colony growth rates varied markedly between day 56 and day 90, with the fastest colonies outgrowing the slowest colonies by two orders of magnitude (Fig. 1.2a). Growth differences could be due to differences in stored energy between queens, genotypic differences of the fungal cultivar

sustaining colony growth, and differences in associated microbiomes that could be beneficial or detrimental to colony function. Fast-growing leafcutter colonies in the field are less prone to raids by neighboring colonies (Rissing et al. 1989), and in *A. texana*, fast-growing colonies should be able to expand the nest faster towards greater depth and then move the incipient garden to more stable temperature and humidity conditions. Initial colony growth rate, dependent on queen factors (capacity to lay eggs and nourish both brood and garden) and on brood development rate (a function of temperature in the top soil and depth of foundress chamber), therefore may be one of the most important components of colony fitness in *A. texana*. The growth rates of the fungus gardens also varied between colonies (Fig. 1.2b). While most gardens increased in volume between days 70 and 90, several remained unchanged or lost volume. Reductions in garden volume occurred most frequently before a colony lost its garden completely.

The relationship between worker production and fungus garden growth was variable during the first 3 months of growth. At day 70, there was a significant, positive relationship between fungus volume and worker number using a linear regression (adjusted $r^2 = 0.456$; df = 21; p = 0.0002; Fig. 1.2c); whereas, at day 90 there was no statistically significant relationship between these two growth variables (adjusted $r^2 = 0.009$; df = 21; p = 0.286; Fig. 1.2d). This suggests a changing relationship between worker production and fungus garden growth in incipient colonies in *A. texana*. Such changing relationships were found in studies of *Acromyrmex versicolor* and *Trachymyrmex septentrionalis*, but at different stages of development (Cahan and Julian 1999; Seal and Tschinkel 2007; Clark and Fewell 2014). For *Acromyrmex versicolor*, Clark and Fewell (2014) found a developmental transition point at week 27 and colony size of 89 ± 9 workers, when the relationship between worker number and fungus garden growth rates switched from a weak, positive relationship to a strong and stable, positive relationship. Seal and Tschinkel (2007) found

a similar pattern in *Trachymyrmex septentrionalis*, in which sexually mature colonies have a much stronger, positive relationship between worker production and garden growth than smaller, incipient colonies. Given the brief, 14-week time period over which we measured growth in our study, it is possible that a similar transition occurs in *A. texana* at a later stage. Overall, there appears to be a trend across these studies that the growth relationship between worker production and fungus garden growth is weak and variable during very early colony development. It is currently unknown if this growth relationship stabilizes in *Atta* colonies as it does in *Acromyrmex* and *Trachymyrmex* colonies at later stages of colony development.

Fungal Disease and Mortality in *A. texana* Queens

Of the 91 queens that we monitored for parasitic fungi, 68 queens died by the end of our study. We could visually distinguish two dominant fungal morphotypes growing on 55 of the 68 dead queens, a white morphotype and a yellow-green morphotype. Twenty-four of the queens were covered in clumps of powdery yellow-green spores; 31 were covered in a dense, white mat of mycelium. We kept collection containers closed until workers began to pupate (about 45 days after mating flight), which prevented cross-contamination by pathogens. It is therefore likely that queens became infected with these pathogens in the field, either prior to the mating flight, during the flight, or when aggregating for nest excavation at our collection sites. All 20 fungal-isolation attempts were successful and isolates appeared free of contaminants because: (1) each isolation attempt yielded only one morphotype per inoculate; and because (2) there was no visible difference between the replicated isolates for each of the two dominant types (i.e., the fungus appearing white on a queen always yielded a white mycelium on plates; the spore-bearing, yellow-green fungus on queens always yielded a spore-bearing, yellow-green mycelium). Cultures were morphologically

highly similar to the two types visible on the queens, suggesting our isolates represent the main fungal morphotypes seen on the dead queens.

DNA sequencing of the ITS gene from one of each of the two morphotypes, identified the two fungi from the dead queens as *Aspergillus flavus* (yellow-green growth) and *Fusarium oxysporum* (white growth). Each of these groups include many species and varieties that cannot be differentiated by the ITS gene. However, in both cases sequence similarity was greater than 98% to respective sequences deposited in Genbank (http://www.ncbi.nlm.nih.gov) and the molecular species identifications were consistent with the observed culture morphology. *Aspergillus flavus* is a pathogen of animals and plants and is a known, sporadic disease of leafcutter ants (Boucias and Pendland 1998; St. Leger et al. 2000; Hughes and Boomsma 2004). *A. flavus* has also been found in the dump piles (expended garden and dead ants) of several leafcutter species, including *A. colombica*, *Acromyrmex echinatior*, and *Ac. octospinosus* (Hughes unpublished, cited in Hughes and Boomsma 2004). Similarly, *F. oxysporum* has frequently been isolated from *Atta* gardens (Rodrigues et al. 2005, 2008). Rodrigues et al. (2010) isolated *F. oxysporum* from dead queens of *Atta laevigata* and *Atta capiguara* in Brazil, suggesting that perhaps this fungus has the potential to cause disease, but it remained unclear whether the death of the queens was specifically due to *F. oxysporum*. Several species of *Fusarium* can infect a diverse range of insects (O'Donnell et al. 2012), and there is no evidence to date that any *F. oxysporum* lineages are primarily or exclusively ant-associated. *A. flavus* and *F. oxysporum* appear to be pathogens that are harmful to the ants, rather than solely to the garden, because queens that became overgrown with these fungi died within the first 9 days after their mating flights, which was before they established their gardens. It is possible that these fungus-overgrown queens were energetically weakened during their mating flights, such that opportunistic pathogens were able to

overwhelm their immune systems in the following days. A more comprehensive investigation of the foundresses' microbial ecology is needed to assign definitive cause of foundress death to either *A. flavus* or *F. oxysporum*.

Observation of *Megaselia scalaris* Feeding on *A. texana*

Megaselia scalaris phorid flies (Phoridae, Diptera) were found with a dead *A. texana* queen on July 9th in its collection container. This queen was collected from Newcastle, Texas, which is the northwestern range limit of *A. texana*. *M. scalaris* feeds on a wide range of decaying organic materials and was likely not the cause of death for this queen, but rather fed on the queen post-mortem (Disney 2008). We can rule out the possibility that *M. scalaris* parasitized this queen, because *M. scalaris* females do not possess the necessary piercing ovipositor to deposit eggs inside a host's body. *M. scalaris eggs* could have been laid on the queen before she was collected; alternatively, it is possible that *M. scalaris* entered the container through an undetected crack in the container housing the queen in the lab, as *M. scalaris* has ''an extraordinary capacity to penetrate into or escape from seemingly closed containers'' (Disney 2008). *M. scalaris* has not previously been found to feed on *Atta* corpses, but has been found feeding on refuse piles of other ant genera (*Eciton*) and moribund termite alates, along with many other sources of decaying organic material (Disney 2008).

CONCLUSION

The first 3 months of the *Atta* colony life cycle are particularly fraught with obstacles for foundress queens and their incipient colonies (Weber 1972; Fowler et al. 1984, 1986). As in other ant species (Cole 2009 and references therein), high risk of mortality during nest-founding and

intense competition between conspecific colonies can amplify small differences in initial growth rates to have drastic effects on colony fitness.

Early growth rate is especially important in determining which colonies will be able to (1) resist execution by other *Atta* colonies (Fowler et al. 1984; Fowler 1987); (2) maintain careful hygiene to cope with diseases of leafcutter ants (Yek and Mueller 2011) and of the fungal garden (Mueller et al. 2010); and (3) expand the colony rapidly to reach a stable microclimate at deeper soil layers (Mueller et al. 2011a, b). The observed variation in colony growth rate (Fig. 1.2) combined with the many obstacles faced by new, small colonies, suggests that early growth rate is likely an important selective factor affecting fitness in *Atta texana*.

Two potential fungal pathogens, *Aspergillus flavus* and *Fusarium oxysporum* infected queens that died before they were able to establish their gardens, suggesting that such pathogens could have detrimental effects on queens during nest establishment. It remains unclear whether the pathogens identified here were the primary cause of death. Experimental exposure of queens to fungal pathogens like *A. flavus* and *F. oxysporum* can determine their virulence and fitness-effects on *A. texana*.

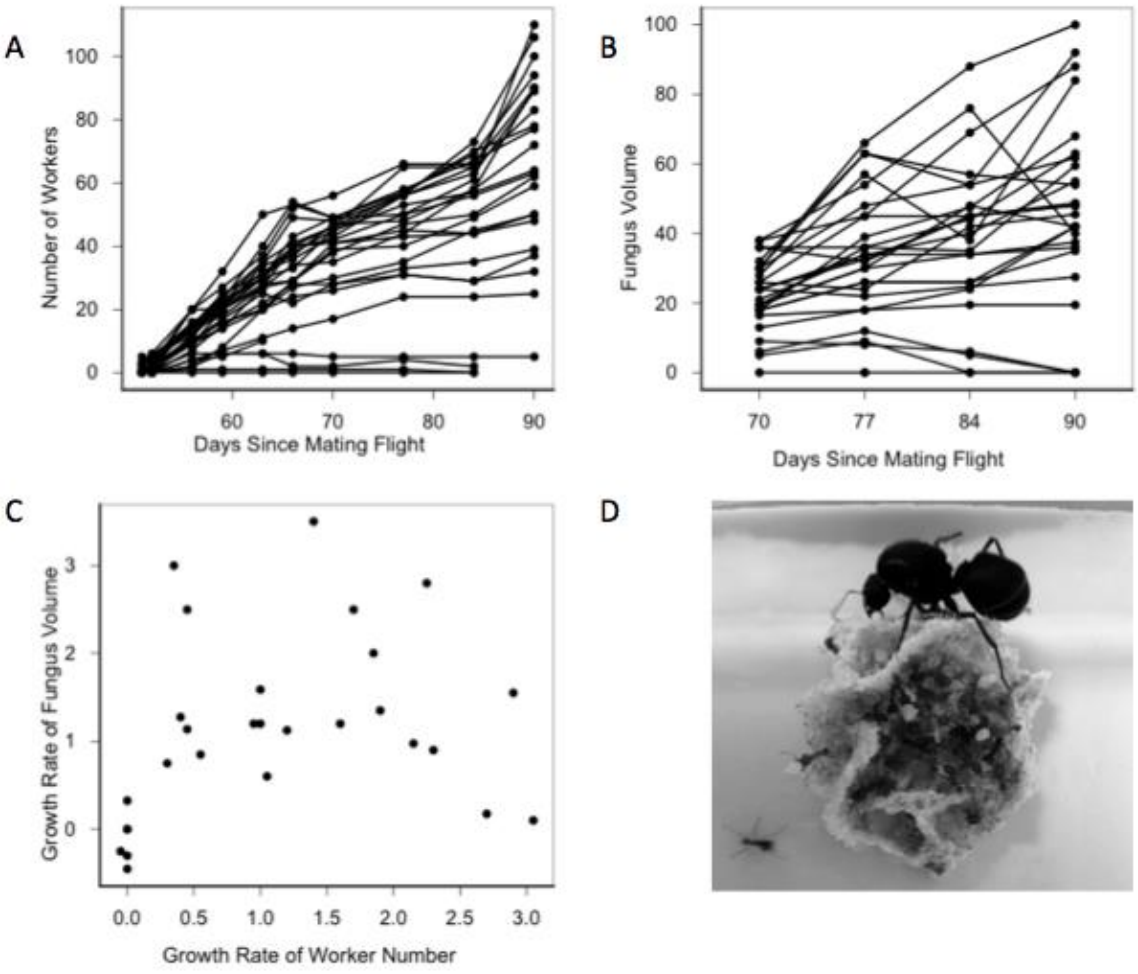

Figure 1.1 **A** Colony growth rates as measured by worker number of *Atta texana* colonies between days 56 and 90 post-mating flight. Each of 28 colonies is represented by a black line. A line that terminates before day 90 represents a colony that had died by that time point. **B** Colony growth rates as measured by fungus garden volume between days 70 and 90. **C** Correlation between the worker number and fungus garden growth rates between days 70 and 90, ($r_s = 0.50$; df = 26; p = 0.0064). **D** *A. texana* colony at day 73. Workers have begun building up the walls of the fungus garden.

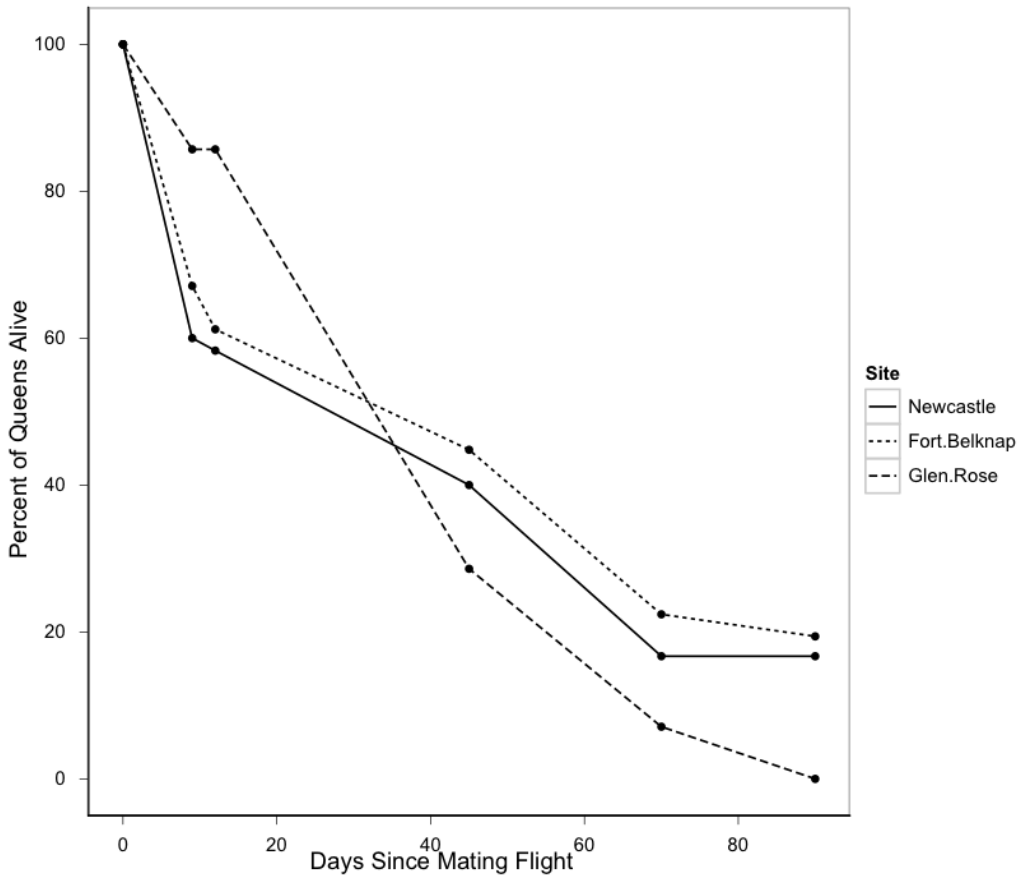

Figure 1.2 Survivorship rates of *Atta texana* foundresses collected on the 25th (Glen Rose, TX, n=14) and 26th (Newcastle, TX, n=60; Fort Belknap, TX, n=67) of May 2014.

Table 1.1 Survivorship rates of *Atta texana* foundresses collected on the 25th (Glen Rose, TX, n=14) and 26th (Newcastle, TX, n=60; Fort Belknap, TX, n=67) of May 2014.

Date	Newcastle, Texas	Fort Belknap, Texas	Glen Rose, Texas	Total
	# Alive			
May 25 (Day 0)	n/a	n/a	14 (100%)	n/a
May 26 (Day 0)	60 (100%)	67 (100%)	n/a	141 (100%)
June 4 (Day 9)	36 (60%)	45 (67.1%)	12 (85.7%)	93 (66.0%)
June 7 (Day 12)	35 (58.3%)	41 (61.2%)	12 (85.7%)	88 (62.4%)
July 10 (Day 45)	24 (40%)	30 (44.8%)	4 (28.6%)	58 (41.1%)
August 4 (Day 70)	10 (16.7%)	15 (22.4%)	1 (7.1%)	26 (18.4%)
August 24 (Day 90)	10 (16.7%)	13 (19.4%)	0 (0%)	23 (13.6%)

Chapter 2: Collective memory persists in leafcutter ant colonies due to communication on the foraging trail and through their mutualistic fungus

ABSTRACT

All memory is a collective process. In the brain, populations of neurons interact and collectively fire to encode, store, and retrieve information. Parallels can be drawn between the interactions of neural populations in the brain and the interactions among group members within other complex biological systems. Collective memory processes can allow a learned behavior to be expressed in a group even when some individuals did not experience the initial stimuli that caused the learning. In this study, we manipulated information regarding leaf quality that could be communicated between different age cohorts of leafcutter ant workers. We found that the collective 'memory' of leaf quality affected both experienced and naïve ants' behavior, as younger, naïve ants conformed to their older nestmates' leaf preference while naïve and experienced workers foraged together. This memory persisted even when younger, naïve ants made up 80-99% of their colonies and made on average 92% of the foraging decisions. However, this collective memory did not outlast the lifespan of the last original ants that experienced that change in leaf quality (i.e., colony memory ceased once experienced ants had died). We found that the foraging choices of naïve ants were strongly related to the types of leaves ants had detected in their fungus garden, and the types of leaves outgoing foragers encountered when interacting with incoming foragers carrying leaves on the foraging trail. These two positive feedback mechanisms likely constitute the mechanism allowing collective expression of memories held by only a subset of ants in a colony. Finally, we found that naïve ants responded differently than experienced ants to the same new cues from their fungus gardens. Naïve ants more readily re-learned, compared to experienced ants, that a once harmful leaf type was again beneficial for their fungus garden. This

example of collective memory highlights the distinction between social learning and social influence and asks how reward or reinforcement from groupmates might play a role in maintaining knowledge within groups across different ecologically relevant timescales.

INTRODUCTION

Memory is operationally defined as the encoding, storage and retrieval of information acquired through experience and learning. It enables behavioral plasticity in animals, allowing them to adapt to changing circumstances within lifetimes. While much work has been done to determine how potential neural circuits enable memory and learning, operational definitions in behavioral sciences do not rely on neuronal evidence (Domjan 2010; Shettleworth 2010). The key diagnostic for learning or memory is to compare two groups, one that has had a particular experience and one that has not (Shettleworth 2010). This definition of learning is focused on outcomes, rather than on mechanisms. In many cases it may be important to make a mechanistic distinction between learning and other forms of behavioral plasticity. However, from an evolutionary or ecological perspective, many different mechanisms could be acted upon to produce the similar outcomes. When making comparisons across levels of biological hierarchy, a dual focus on similar outcomes and their mechanisms can lead to fruitful insights across disciplines.

For instance, behavioral attempts at creating a broadly functional definition of memory from a strictly neural perspective are challenged by instances where behavioral plasticity and the maintenance of information are found in systems that do not possess neurons [e.g. chemical habituation in slime molds (Boussard et al. 2019); intergenerational epigenetic memory encoded by histone methylation (Turner 2002); 'reinforced learning' in artificial intelligence systems (Busoniu et al. 2010)]. Also, in human organizations, the concept of 'organizational memory'

refers to the ability of institutions to encode, store, and retrieve information as people cycle through, regardless of how this is mechanistically accomplished (Walsh and Ungson 1991).

From animal species at different levels of biological hierarchy to machine learning, we find analogous processes of learning and memory. Research on learning and memory in these different systems can be reciprocally informative even when the mechanistic basis differs (Reid et al. 2012), that is, information does not rely on neuronal encoding (Domjan 2010). To understand the evolution of behavioral adaptation and the process and function of learning and memory across types and levels of organization, it is thus useful to study systems in which memory is formed and maintained in different ways.

Collective memory is a particularly interesting example. Many biological systems are essentially 'groups' of units at a lower level of organization, but have evolved adaptive behavior at the system level: e.g. genomes made of multiple interacting genes (Maynard-Smith and Szathmary 1995), Myxobacteria (Shimkets 1999), *Dictyostelium* slime molds (Bonner 2003), volvocine algae (Herron and Michod 2008), multicellular organisms (Grosberg and Strathmann 2007), social insect colonies (Seeley 1997), and mole rats (Burda et al. 2000). These systems often possess many traits that we ordinarily think of as individual-level traits at the group level, such as 'personalities' (Wright et al. 2019), different life-history strategies (Bengston et al. 2017), territoriality (Lacey and Sherman 1991), and the ability to make decisions (Visscher 2007; Ross-Gillespie and Kümmerli 2014). Do such collective systems also 'learn' and possess 'memory' (Biro et al. 2016; Berdahl et al. 2018)?

Social insects offer interesting opportunities for the study of collective memory. First, they are selected at the colony level and thus individuals benefit more from information sharing, and have evolved more sophisticated and information-rich communication systems than any other non-

human animals (Wilson and Holldobler 2009). For this same reason, they may have also evolved mechanisms of nestmate interaction to conserve important information for the colony – i.e., to promote 'memory' maintenance. Second, often the lifespan of colonies is much longer than that of workers (Giraldo and Traniello 2014). It is thus possible that colonies would benefit from preserving information for longer than an individual lifespan. Lastly, social-insect colonies have been heralded as great study systems for collective cognition generally (Seeley 1995; Couzin 2009; Razin et al. 2013). In particular, in both consensus decision making and allocation decisions such as in foraging, information exchange and use has been extensively investigated and can be highly complex, involving both signals and cues (Leonhardt et al. 2016). In most cases however, communicated information is short-term (i.e., relevant on a scale of minutes or hours, although see 'modulatory communication' e.g. in honey bees; Schneider and Lewis 2004).

The mechanisms by which collective memory is formed (i.e., information encoded) and maintained (i.e., information stored) and forgotten (i.e., decay of information) are particularly interesting as they may give clues as to how behavioral plasticity can be maintained at any level of organization. Memory processes rely on the complex interactions between individual neurons. While advancements in neurophysiology have improved our understanding of memory encoding and storage, there are many aspects of these interactions that remain mysterious. Biological models of collective cognition, like ant colonies, have served as inspiration for improving recurrent neural networks (Desell et al. 2015). These models are much easier to manipulate directly and visualize than neural interactions, and may be key in elucidating important aspects of the maintenance of information within complex systems.

In groups, memory could simply be stored in individual brains, and thus affect the group outcome through the experienced individuals' behavior. Alternatively, information could be

shared via social learning (Whiten et al. 1999; Jesmer et al. 2018), and possibly retained for the long term via continued social information transfer to new individuals (Rosengren and Fortelius 1986; Schofield et al. 2018). Information can also be stored in the environment, as in physical and chemical foraging trails in insects (Czaczkes et al. 2015) and slime molds (Smith-Ferguson et al. 2017), in food stores (Johnson 1991; Johnson et al. 1994; Dornhaus and Chittka 2005), or in built structures (Grasse 1959). This environmentally-stored information can affect colony behavior and cause behavioral plasticity in the same way as conventional (i.e., neuronally stored) memory does. One way to discover how these 'memories' are stored is to test when and how the memory is forgotten. Memories within a group could be forgotten or otherwise lost due to 1) neuronal forgetting by the individuals who had the experience, 2) death of individuals who had the experience, 3) decay of information stored in the environment, or 4) not at all. Here we study how individual memory, worker turnover, and nestmate communication contribute to longer-term collective information storage about stable food sources.

Leafcutter ants collect leaves which they use as substrate to grow gardens of fungus in underground chambers. The ants and their fungus are obligate mutualists; the fungus metabolizes leaf material provided by the ants and the ants eat the mycelium of the fungus. Tropical leafcutter ants collect leaves from as many as 50-80% of the plants in their foraging range (Cherrett 1989) and bring them to their fungus gardens, which provide feedback to the ants on the quality of the leaves they have collected (Herz et al. 2008). Leaf age (Littledyke and Cherrett 1978), leaf texture (Cherrett 1972; Waller 1982), chemical composition (Littledyke and Cherrett 1978; Hubbell et al. 1983; Thiele et al. 2014), and endophyte load (Bittleston et al. 2011) are all characteristics that impact leaf suitability for fungus garden growth that can vary across a spatial landscape or even over the course of a season (Aide 1993; Reich et al. 2004). Leafcutter ants also change their

foraging patterns to reflect changing colony nutritional requirements depending on brood, temperature, or moisture conditions within the nest (Lewis et al. 1974; Rockwood 1976; Shik et al. 2016).

Laboratory and field studies have shown that leafcutter ants learn to reject species of plants whose leaves are detrimental to fungus garden growth (Herz et al. 2008; Saverschek et al. 2010a). Rejection is based on cues from their fungus gardens that are produced for the 2-3 days after harmful leaves have been incorporated into a garden (Herz et al. 2008). After learning to reject a plant species, leafcutter ants colonies have been shown to continue to avoid this species of plant for as long as 30 weeks in the laboratory (Ridley et al. 1996) and for 12-18 weeks in the field (Saverschek et al. 2010a). In these experiments, otherwise suitable leaves were treated with a fungicide at just one time point and later are offered to the ants untreated and no longer harmful. Leaf rejection persists for many weeks without any further reinforcement that the previously treated leaf type is still harmful, and indeed it is no longer harmful in these experiments. It is likely that new workers emerge and mature to foraging age during this time, and these workers are naïve to which leaves had been treated, that is, they would not have themselves experienced cues from the fungus designating the previously treated leaf type as harmful.

Arenas and Roces (2016b, a, 2017, 2018) have demonstrated that ants learn about the suitability of different types of leaves inside the nest, in both the chambers containing fungus garden and chambers containing waste. Waste is generated by leafcutter ant colonies as old and damaged bits of fungus garden are ‘weeded out’ and thrown away (Fowler and Louzada 1996). Some species keep their waste in specific underground chambers, while other species deposit their waste in above-ground middens (Farji-Brener et al. 2016). When a colony is given cycloheximide-treated leaves, waste generation increases 16-28 hours after the treatment (Arenas and Roces

2016b). During this time, bits of damaged fungus garden mixed with partially decomposed leaf material are brought by gardening ants to the trash. Leaf rejection information can be learned and remembered solely from this waste material. Here, we ask whether worker turnover process impacts colony leaf preferences in the absence of any possible rejection cues from the waste.

We investigate the collective memory of leaf suitability by asking the following specific questions: 1) Will naïve ants display leaf rejection they could not have learned directly from their fungus garden or waste dump? 2) When will colony-level rejection be forgotten relative to the mortality of the original, experienced cohort of workers? 3) If naïve ants do display leaf rejection, how might they acquire this behavior? 4) Do naïve and experienced ants differ in their tendency to use social information? To address these questions, we allowed colonies to learn that one of two leaf types was unsuitable for their fungus garden (using treatment with cycloheximide, a fungicide). We conducted four different treatments, which varied also in whether ants could incorporate these (now harmless) leaves in their garden to update their information (Fig. 2.1 & Table 2.1). Manipulating leaf incorporation allowed us to distinguish between a process of re-learning with new information from the fungus garden, from a process of forgetting in the absence of new information from the garden.

METHODS

Colony Collection and Laboratory Maintenance

We excavated 48 queenright *Atta colombica* colonies in Gamboa, Panamá, between February 2017 and February 2018. The colonies we collected included a range of sizes, but most were incipient colonies with 1-3 nest entrances (Weber 1972) and none were very large (more than 5 entrances). We kept colonies in a laboratory with large windows and natural light conditions,

temperatures fluctuated between 23-26°C. Colonies were housed in plastic nest boxes covered with glass lids, which varied in size according to the size of the colony (small: 7x7x3 cm^3; medium: 9x13x5 cm^3; large: 11x7.5x16.5 cm^3). We poured a layer of hydrocal plaster in the bottom of each nest box and moistened the plaster with deionized water as needed to maintain them near 100% humidity. Nest boxes were placed in larger plastic boxes (15x30x12 cm^3 or 25x30x15 cm^3) with walls lined with mineral oil or Insect-a-slip (Bioquip) to prevent escape. Colonies were fed a diet of organic oatmeal flakes, leaves, and flower petals from plants growing in Gamboa, Panamá, primarily *Mangifera indica*, *Adenanthera pavonina*, and *Hibiscus* leaves, and petals from *Lagerstroemia speciosa*. When we started experiments, colonies ranged in size from 31-577 workers, not counting the smallest of garden workers (hereafter referred to as minims, head width < 1mm). All colonies had one queen, though not always the original queen, 7 of the 48 colonies were requeened over the course of the experiment due to queen mortality. New queens were obtained from other incipient colonies and were added to colonies in the experiment following queen mortality. We controlled for any possible effect of requeening in the first model described in Table 2.3 by including it as a random effect in the model. All colonies also contained the fungus and brood, they were found with at the time of collection.

Leaf Preference Tests

All leaf preference tests used *Ipomoea batata* (sweet potato, an herbaceous perennial vine) versus *Lagerstroemia speciosa* ('Pride of India', a tropical tree). Leaves of *I. batata* were collected from plants we grew outside. *L. speciosa* leaves were collected from multiple trees growing in the town of Gamboa. Both types of leaves were collected fresh every day. Neither type of leaf was given to colonies between preference tests. To assess colonies' leaf preferences, we tested them

using the following protocol both before and after we offered treated leaves (see below) to the colonies.

We gave each colony a 11 cm long popsicle-stick bridge which led to a 5x6 cm^2 foraging platform where we placed four freshly cut leaf discs (6 mm in diameter), two discs of *I. batata* leaves and two discs of *L. speciosa* leaves, along the far edge of the foraging platform in alternating order (see Fig. 2.2). Every time a leaf disc was taken by an ant, we replaced that disc with another disc of the same plant species. We calculated leaf choice for each 30-minute test for experienced and naïve ants separately. Leaf choice is calculated as the proportion of collected leaves that were of the previously treated type. We use the term 'previously treated leaf type' to refer to the leaf type that was offered to colonies on Day 0 regardless of whether or not the leaf was actually treated on Day 0. For example, in Treatments II and IV colonies who received untreated *I. batata* discs on Day 0 have *I. batata* as their designated 'previously treated leaf type'. See Table 2.1 and Fig. 2.1 for Treatment details. Tests lasted for 30 minutes and were video-recorded using a Canon EOS Rebel T6i digital camera. Researchers who scored these videos were blind to treatment.

Induced Leaf Rejection with Cycloheximide Treatment

We treated leaf discs with a low concentration solution of cycloheximide (0.03% w/w) through suction infiltration (detailed methods in Herz et al. 2008). When ants collect cycloheximide-treated leaves and incorporate them into their fungus gardens, they learn to reject leaves from that species of plant (Herz et al. 2008; Saverschek et al. 2010a; Saverschek and Roces 2011).

Treatments and Testing Schedule

We tested the contributions of individual memory and worker interactions to the maintenance of a putative collective memory with a 2x2 factorial design that included four treatments (Table 2.1 & Fig. 2.1). These four treatments varied in whether colonies were offered leaves treated with cycloheximide to induce leaf rejection and whether colonies were allowed the possibility to update their information regarding leaf quality by incorporating leaves of the previously treated (but now harmless) type into their fungus gardens after Day 1 of the experiment. This second variable of leaf removal allowed us to compare the dynamics of collective memory and forgetting when re-learning from the fungus garden was and was not possible.

We determined when and if experienced ants forgot the leaf rejection they learned from their fungus garden by comparing their leaf preferences in colonies who did and did not receive cycloheximide treated leaves. We determined whether naïve ants behaviorally expressed the rejection memory of experienced ants by comparing their leaf preferences in colonies wo did and did not receive cycloheximide treated leaves. We determined whether experienced or naïve ants responded to new information from their gardens by comparing leaf preference in colonies who could and could not incorporate leaves of the previously treated type in their gardens throughout the experiment.

We used the same dataset to test four possible mechanisms of social information transfer from experienced to naïve ants, and one possible effect of our leaf removal methods on ants' leaf preferences. Specifically, we used video-recordings of preference tests to measure the effect of outbound contacts between foragers, inbound contacts between foragers, and the presence of minims on the leaf choices of individual foragers (See Table 2.2). We also measured the proportions of both types of leaves that ants incorporated into their gardens, to test for the effects

of past leaf incorporation on future leaf choice (See Table 2.2). Lastly, we tested whether repeatedly removing leaves of the previously treated type decreased ants' likelihood to collect leaves of that type, by two-week age cohort (See Table 2.2).

We conducted 2 similar experiments over the course of two years. Experiment 1, consisted of Treatments I and II, was conducted in 2017, while Experiment 2, consisted of Treatments III and IV, was conducted in 2018. When we discuss our results below, we are clear to specify the one instance in which a between-year comparison was made.

Experiment 1:

Experiment one consists of Treatments I and II. On Day 0 of the experiment we gave colonies in Treatment I 15 leaf discs of *I. batata* which we treated with cycloheximide. On the same day, we gave colonies in Treatment II 15 untreated leaf discs of *I. batata* (see Table 2.1 for Treatment specifics).

We tested all colonies' leaf preferences on Day 8 and subsequently every two weeks (Day 21, Day 35, Day 49 etc.). We continued testing each colony until all original, experienced ants in that colony had died off and had been replaced by newly emerged ('naïve') workers. For all colonies and all preference tests after the treatment occurred, we offered both types of leaves untreated.

For colonies in Treatments I and II, we allowed ants to incorporate *L. speciosa* leaf discs (the leaf type that was never treated) into their gardens during all leaf preference tests. But, when ants carried *I. batata* leaf discs (the leaf type that was treated on Day 0) toward their nest chambers we removed those discs gently with forceps immediately before the foragers carrying them entered their nest chamber in all preference tests after Day 0 (i.e., when those leaves were no longer

treated). We removed *I. batata* discs to ensure that any change in leaf preference was not due to the ants directly re-learning leaf acceptance from their fungus gardens once *I. batata* discs were no longer treated or harmful.

Experiment 2:

In our second experiment we followed the same testing schedule as in the first experiment. Leaves were offered (cycloheximide treated, or not) on Day 0, and leaf preference tests were performed on Day 8 and every two weeks thereafter.

When we began Experiment 2 we noticed that colonies sometimes need a second day of treatment to ensure that the ants learned the rejection, this was not the case in 2017. To be sure all cycloheximide treated colonies initially learned the rejection we offered twice as many leaf discs on Day 0. And, on Day 1 we offered leaf discs again (treated or untreated) to be sure colonies had learned the rejection before proceeding with the experiment.

Colonies in Treatment III received cycloheximide treated leaves on Day 0. For these colonies we allowed ants to carry all chosen leaf discs into their gardens and to incorporate either type of leaf discs for the first two hours following each preference test. After two hours had elapsed we removed all leaf discs that had been carried into the nest chamber but had not yet been incorporated into the fungus garden. Colonies in Treatment IV never received cycloheximide treatment. Every other aspect of Treatment IV matched that of Treatment III.

Measuring the Duration of a Worker Turnover Cycle

To track the process of worker turnover, on Day 7 of the experiment, we marked all ants (both older workers and newly-eclosed ants) with the same color as these workers all may have directly encountered fungal cues designating the treated leaf type as harmful to their fungus gardens (Herz et al. 2008). All ants painted on or before Day 7 will be referred to as experienced ants, meaning that they might have had direct experience with fungal cues regarding leaf suitability on Day 0, in colonies who were offered cycloheximide-treated leaves. However, for comparison, ants painted before Day 7 are referred to as experienced, and ants eclosed after Day 7 as naïve, regardless of treatment.

Subsequently, every two weeks we dissected each colony's fungus garden to count the numbers of ants in each age group, to repaint those ants whose markings had been partially chipped away, and to paint the newly-eclosed ants. All ants with no detectable paint markings that were not callow were removed from the colonies. This was a small proportion of workers, fewer than 5% of the number of painted ants. For Treatments I and II, we painted newly-eclosed ants with a new color every two weeks to follow cohorts of workers in two-week intervals. For Treatments II and IV, we marked ants that eclosed before Day 7 in one color and all ants who eclosed after Day 7 with a second color.

All ants were marked with two dots of paint from a non-toxic, oil-based Sharpie™ paint marker, model 1770459. We applied paint to the dorsal surface of the head and thorax of each ant using a thin piece of wire. A colony's worker turnover cycle was designated as the number of weeks elapsed until all original, experienced workers had died and had been replaced by newly-eclosed, naïve workers.

Waste Removal

We removed all waste and cleaned plastic ant-housing containers after each time we dissected the fungus gardens and painted ants (every two weeks). As a result, the ants we are calling naïve never came in contact with trash containing bits of damaged fungus mixed with treated leaf particles.

Measuring Past Leaf Incorporation

Past leaf incorporation was calculated as the proportion of the leaf discs incorporated that were of the previously treated leaf type, over the time from the first test following the treatment at Day 0 to the test immediately before the time point in question.

Statistical Methods

We used 4 generalized linear mixed models (GLMMs) to tests the effects of cycloheximide treatment, leaf removal pattern, and past leaf incorporation on the leaf choices of naïve and experienced ants (details in Table 2.3). We included cycloheximide treatment, leaf removal pattern and past leaf incorporation as fixed effects, representing the direct personal experience with (bad) leaf quality, and inferred information about leaf quality from the fungus garden. We included colony ID, week, the type of leaf that was treated, and whether or not a colony had ever been requeened as random effects.

We also used a GLMM to test the effects of outbound contacts, presence of minims, and previous attempts collecting *I. batata* on the binary leaf choices of individual foragers. We included colony ID and week as random effects. We used the 'lme4' package (Bates et al. 2015).

We used the 'emmeans' function in the R package 'emmeans' to calculate effect sizes as the estimated marginal means (Lenth 2020). To report percentage differences in the collection of

leaf discs of the previously treated type, we divided the estimated marginal mean by the average number of discs of the previously treated type taken by the group in question. All statistical analyses were performed in R 3.6.1 (R Core Team, 2019).

RESULTS

1. Will naïve ants also reject leaves without ever personally experiencing negative cues from their fungus garden?

We saw that naïve ants rejected leaves of the previously treated type, consistent with the hypothesis that information about which leaves to reject is directly or indirectly passed on to new colony members. Using a generalized linear mixed model (GLMM) we found that naïve ants in cycloheximide treated colonies collected an estimated 28% fewer leaf discs of the previously treated type, compared to untreated colonies with the same pattern of leaf removal (Treatments I and III versus Treatments II and IV: effect size in number of leaf discs = 3.27, $z=4.36$, $p<0.001$, df = 206; Fig. 2.5).

2. How long can collective memory of leaf quality persist relative to the lifespan of worker ants?

Naïve ants in cycloheximide-treated colonies rejected leaves of the previously treated type even when naïve ants made up 80-99% of their colonies. Considering only tests where the proportion of naïve ants is between 0.8 and 0.99, naïve ants in cycloheximide treated colonies collect an estimated 37% fewer leaves of the previously treated type than naïve ants in untreated colonies with the same pattern of leaf removal (effect size in number of leaf discs: 5.54, $z = 4.28$, $p < 0.001$, df = 205; Table 2.5). When naïve ants made up 80-99% of the colony, they made on average 92 ± 14% of the foraging decisions.

However, by the time the worker turnover cycle was complete (i.e., all experienced ants had died and been replaced by naïve ants), colonies' leaf choices were not significantly different across treated and untreated colonies (Treatments I and III versus Treatments II and IV: effect of

cycloheximide treatment on leaf choice when the proportion of naïve ants is 1.0, $z = 0.015$, $p = 0.99$, $df = 36$; Fig. 2.3). On average the worker turnover process took 17 weeks, turnover times ranged from 5 to 29 weeks among colonies. This result supports the hypothesis that in this case collective memory and forgetting is driven by worker interactions and worker mortality, rather than solely individual memory and neuronal forgetting. Also, while naïve ants' behavior is affected by their nestmates' past experiences, naïve ants do not retain neuronal memory that could have been transferred via social learning.

3. If naïve ants do display leaf rejection, how do they acquire this behavior?

We tested five hypothetical mechanisms of social information transfer (Table 2.2 and Fig. 2.2) using data we collected from our preference test videos. Our data support both hypotheses concerning positive feedback mechanisms and reject all three hypotheses concerning negative feedback mechanisms.

A) Past leaf incorporation: If naïve ants' preferences are shaped by which leaves they detect in the garden, we should see a positive relationship between the amount of leaves of the previously treated type collected by naïve ants and the amount of that leaf type that had been incorporated in the garden since they eclosed. This was the case. Using a GLMM, we found that naïve ants in colonies that had incorporated more leaves of the previously treated type collected a greater proportion of leaves of that type ($z=2.61$, $p=0.009$, $df=98$; Table 2.3). But the same was not true for experienced ants ($z=0.09$, $p=0.93$, $df=109$; Table 2.3).

B) Outbound contacts: If naïve ants prefer leaves they encounter on the foraging trail carried by other ants, we should see that ants who contact a higher proportion of leaves

of the previously treated type will be more likely to collect that same type of leaf. This was true. Ants were more likely to collect a particular type of leaf when they contacted nestmates carrying that type of leaf more frequently ($z=2.21$, $p=0.027$, $df=258$; Table 2.4).

C) Cues from minims: Minims (head width < 1mm) did aggregate on and around leaves of the previously treated type in cycloheximide-treated colonies more often than on the other leaf type in the same colonies (Wilcoxon test, $W=27211$, $p<0.001$, $n=400$), or the same leaf type in colonies that received no cycloheximide treatment (Wilcoxon test, $W=32902$, $p<0.001$, $n=508$). If minims discourage naïve ants from collecting certain leaves, we should see that when there are more minims on leaves of the previously treated type, foragers will be more likely to collect the opposite type of leaf. This was not true. A forager's leaf choice did not depend on the number of minims standing on leaves of the previously treated type at the moment of her decision ($z=-0.79$, $p=0.43$, $df=258$; Table 2.4; Fig 2.6).

D) Inbound antennations: If naïve ants receive negative feedback about the leaf type they are carrying back to the nest via antennation from experienced ants, we might see that naïve ants carrying leaves of the previously treated type receive more antennations in cycloheximide treated colonies. This was not the case. In fact, ants returning home with leaves of the previously treated type received fewer antennations on their inbound journeys than did ants carrying the other leaf type in those same colonies (Wilcoxon test, $W=3684$, $p=0.003$, $n=198$), or ants carrying the same leaf type in colonies that received no cycloheximide treatment (Wilcoxon test, $W=4133$, $p=0.02$, $n=203$).

E) Forceps removal of leaf discs: If experimentally removing carried leaves with forceps changes the preference of ants that carried them, we should see a negative relationship between the number of times leaves of the previously treated. This was not true. We found no relationship between the number of times leaves of the previously treated type we removed with forceps and the amount of those leaves that ants collected (z=0.42, p=0.68, df=258; Table 2.4).

4. Do naïve and experienced ant differ in their tendency to use social information?

To address whether naïve ants were more likely to use new social information (from the fungus garden) than experienced ants, we tested whether either group would collect more leaves of the previously treated type in colonies that could incorporate both leaf types in their garden (and subsequently learn that neither leaf type was harmful), compared to the same group in colonies that could not incorporate leaves of the previously treated type.

Naïve ants in Treatments III and IV collected an estimated 41% more leaves of the previously treated type than naïve ants in Treatments I and II (Treatments I and II versus Treatments III and IV: effect size in number of leaf discs = 4.84 , z = 6.33, p <0 .001, df = 206; Table 2.3; Fig. 2.5). Although they had access to the same fungal cues, the same was not true for experienced ants (Treatments I and II versus Treatments III and IV: effect size in number of leaf discs = 0.39, z=-0.46, p=0.65, df=254; Table 2.3; Fig. 2.4). There was no relationship between the leaf preferences of the experienced ants and whether they were allowed to incorporate leaves of the previously treated type or not. These are the only results that depend on between year comparisons.

If naïve ants are more readily able to unlearn collective leaf rejection, compared to experienced ants, we should also see that the same trends exist considering finer scale variation in leaf incorporation. This was the case. When considering only the two treatments where incorporation of leaves of the previously treated type was allowed (Treatments III and IV) we found a positive relationship between past leaf incorporation and leaf preference for naïve ants but not for experienced ants (Treatment III and Treatment IV: effect size in number of leaf discs = 5.21, z=2.61, p=0.009, df=98; Table 2.3). In our model testing the effects of past leaf incorporation and cycloheximide treatment in naïve ants from Treatments III and IV, we found that past leaf incorporation was significant (see above), but cycloheximide treatment explained no additional variation that was not explained by past leaf incorporation (Table 2.3). So, differences in leaf incorporation performed primarily by experienced workers in treated and untreated colonies accounted for the differences in leaf choice by naïve ants.

DISCUSSION

We studied the rejection of previously toxic leaves by experienced and naīve ants and thus the maintenance of information about leaf quality, in *Atta colombica* ant colonies. We found that this 'collective memory' depended on the presence (and thus longevity) of the experienced workers, and thus appears to be stored in these workers' brains. However, the expression of the 'memory' (i.e., the leaf rejection) is shown by both naïve and experienced ants. The collective behavior is thus truly expressed by the colony, not only by workers who had personal, direct experience with the initial stimuli. This phenomenon is likely driven by the interactions between naïve and experienced workers and their differential use of new information from their garden. The collective memory was expressed even in the absence of information transmitted through the

waste, which is another way leafcutter ants learn about leaf quality (Arenas and Roces 2018). The prior experience of older workers was amplified within colonies because ants collected more discs of a particular leaf type when their nestmates carried more discs of that type on the foraging trail. In addition, naïve ants collected fewer leaf discs of the previously treated type when experienced ants did not incorporate that leaf type in the fungus garden. We did not find evidence of persistent social learning, as naïve ants did not retain their socially 'learned' preferences after all experienced workers had died. Finally, we found that experienced ants were less affected by new social information than were their naïve nestmates.

How were naïve ants influenced by their experienced nestmates?

Naïve ants detected what types of leaves were carried by nestmates and what types were incorporated in their gardens, and showed a preference for these previously detected leaf types (see Results, 'outbound contacts' and 'past leaf incorporation'). Both of these processes involve positive feedbacks as ants are recruited to collect leaves that are known to be suitable. Similar positive feedback mechanisms regulating food choice are widespread in social insects (Wenner et al. 1969, Dornhaus and Chittka 1999). Arenas and Roces (2018) found that leafcutter ants learn a preference for scents encountered in the fungus chamber and an aversion to scents encountered in the dump (Arenas and Roces 2018). Similarly, other studies have also found that leafcutter ants are more likely to collect the food they detect their nestmates carrying home on the foraging trail (Roces 1990, Roces 1994, Farji-Brener et al. 2010). Interestingly, our experiment shows that these preferences do not persist in the presence of conflicting information from the fungus garden. But, when leaf rejection is learned directly from the garden, rejection behavior did persist in experienced ants even in the presence of conflicting information from the fungus garden.

We rejected all three hypotheses that naïve foragers learned about leaf suitability via negative feedback mechanisms. Foragers carrying "bad" leaves home did not receive more antennations from nestmates on their inbound journeys. Puzzlingly, minims aggregated on "bad" leaves, but their presence did not appear to deter larger foragers from picking up those leaves. In addition, repeatedly taking leaves away from foragers with forceps did not appear to dissuade foragers from collecting that type of leaf again.

It is also possible that larval learning could contribute to the leaf rejection we see from naïve ants. Some insects can remember a learned association across the neural reorganization that takes place during metamorphosis (Blackiston et al. 2008). Leafcutter ant larvae spend their time in the fungus garden and are fed fungal mycelium. Larvae might detect rejection cues from the garden during the critical 2-3 days post fungicide treatment when those cues are present. However, it would be only a small fraction of our 'naïve' ants that would have been larvae at the time when the rejection cue from the fungus was present. If larval learning were the only mechanism of leaf rejection in naïve ants, we would expect to see no leaf rejection from the first cohort of naïve ants, because these ants were in the pupal stage (10-14 days in *Atta spp.* Marti et al. 2015) when their garden was producing leaf rejection cues. In our study the first cohort of naïve ants did show as much, if not more, rejection of the leaves of the previously treated type as did cohorts of naïve ants that eclosed later. This rules out larval learning as the sole mechanism.

How is the memory of independently reinforced information different from the memory of social acquired information?

We found that naïve ants changed their behavior in accordance with new information available from the fungus garden. But experienced ants were "stubborn" when faced with new,

contradictory information because these experienced ants did not change their behavior (i.e., their leaf choices were not affected by new positive cues from the fungus garden). As a result, our data support the hypothesis that the apparent leaf rejection performed by naïve, unreinforced ants is different than that of ants that personally experienced leaf rejection reinforcement from their fungus garden. Therefore, the temporary persistence of leaf rejection behavior by naïve ants must be understood as a different kind of 'memory' than that displayed by experienced ants, if we are to understand it as memory at all. In these colonies two groups of ants perform the same behavior (leaf rejection) but based on different sources of information. Older, experienced ants act on their memory (private information), whereas young, naïve ants act on social information. It is this distinction that clarifies the concept of collective memory as being separate from individual memory in this case. When colonies were composed of 100% experienced ants, memory of leaf rejection behavior was presumably stored in the brain of each ant who expressed that behavior. When colonies were composed of 80-99% naïve ants, memory of leaf rejection behavior was stored in the brains of experienced ants only. Because naïve ants also expressed leaf rejection behavior, we know that persistence of this collective behavior was due to more than individual memory of any group of ants. Rather, the collective memory was due to both the individual memory of experienced ants, and the interactions between naïve and experienced ants, both direct and mediated through their fungus garden.

What is the benefit of collective memory?

The expected benefit of maintaining memory for any organism would depend on the rate of environmental change (Dunlap and Stephens 2012); this is likely true also for collective memory. In leafcutter ants, environments change throughout the seasons (e.g., dry and rainy seasons), such

that colonies experience seasonal variation in leaf suitability. Leafcutter ants display seasonal avoidance of some leaf types (Fowler and Robinson 1979; Waller 1982). Waller (1986) reports a seasonal schedule under which *Atta texana* colonies opened and closed foraging tunnels leading to specific plants at nearly the same dates every year over the course of five years. Knapp and colleagues (1990) report delayed rejection of several species of untreated leaves by *Acromyrmex octopsinosus*. These natural observations of fluctuating leaf acceptance show that the environment of which leaves should be recognized as suitable or unsuitable might change on a timescale of a few months.

Given the length of collective memory we found here, roughly equal to the length of one worker turnover cycle (median = 17 weeks, range = 5-29 weeks), it is feasible that this system of collective memory allows leafcutter ant colonies to efficiently exploit leaves whose characteristics change over the course of a season. If there is selective pressure for leafcutter ants to retain leaf rejection over the course of a season, and a need to resample leaves and remain behaviorally flexible between seasons, worker turnover rate and a temporary influence of experienced workers on naïve workers could accomplish this task. Furthermore, if a leaf was still harmful after naïve ants began to forage, the temporary and partial influence of experienced ants could ensure that the leaf is resampled only gradually, thereby protecting the fungus garden from collapse.

In conclusion, we have shown how social information sharing can lead to a flexible collective memory at the colony level. This example of collective memory highlights the distinction between social learning and social influence, and asks how reward or reinforcement from groupmates might play a role in maintaining knowledge within groups across different ecologically relevant timescales. Finally, we found two positive feedback mechanisms at work to maintain colony-level leaf preferences. This redundancy of two mechanisms, along with the lack of support for negative

feedback mechanisms, suggests that it may be more beneficial (or simply easier) to collectively remember what is good than to remember what is bad. In fact, despite a robust 'negativity bias' in human and non-human animals in the realms of learning, attention, and affect contagion, memory processes alone are biased toward positivity (reviewed in Rozin and Royzman 2001). Commonalities between individual and collective memory processes, such as a positivity bias, could be fruitful avenues for research on general properties of diverse memory systems.

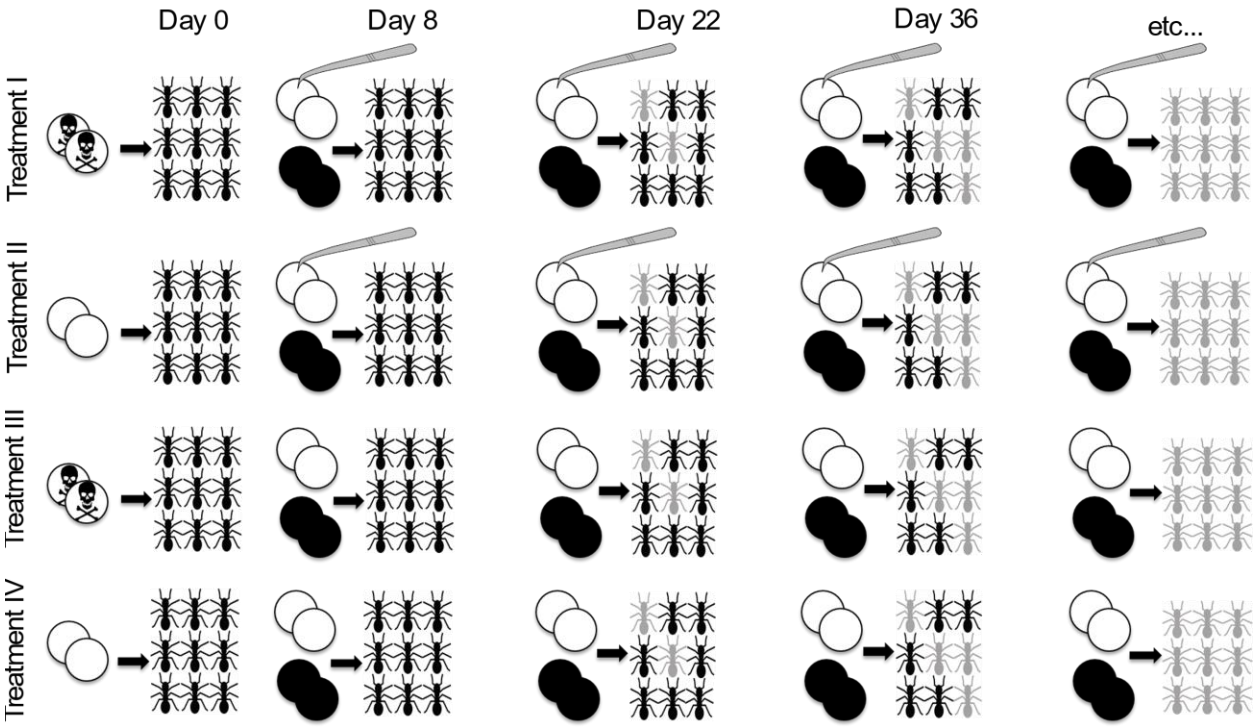

Figure 2.1 Diagram of treatments and testing schedule. Treatments I and III received cycloheximide treated leaves on Day 0. Treatments II and IV received the same type of leaf but without cycloheximide. Leaves with cycloheximide treatment are represented as white discs with skull and crossbones. All treatments received only untreated leaves during all tests after Day 0. White circles represent discs of the previously treated type. Black circles represent discs of the alternate leaf type. For Treatments I and II we removed leaves of the previously treated type before foragers could bring them into their fungus chamber. Grey forceps represent this leaf removal process in Treatments I and II. For Treatments III and IV we allowed foragers to bring both leaf types into their fungus chamber and incorporate both types in their fungus gardens. We re-painted and tested the leaf preferences of all colonies on Day 8 and then every 14 days until all original, experienced ants (black) had died and had been replaced by naïve ants (grey). This process took 17 weeks on average.

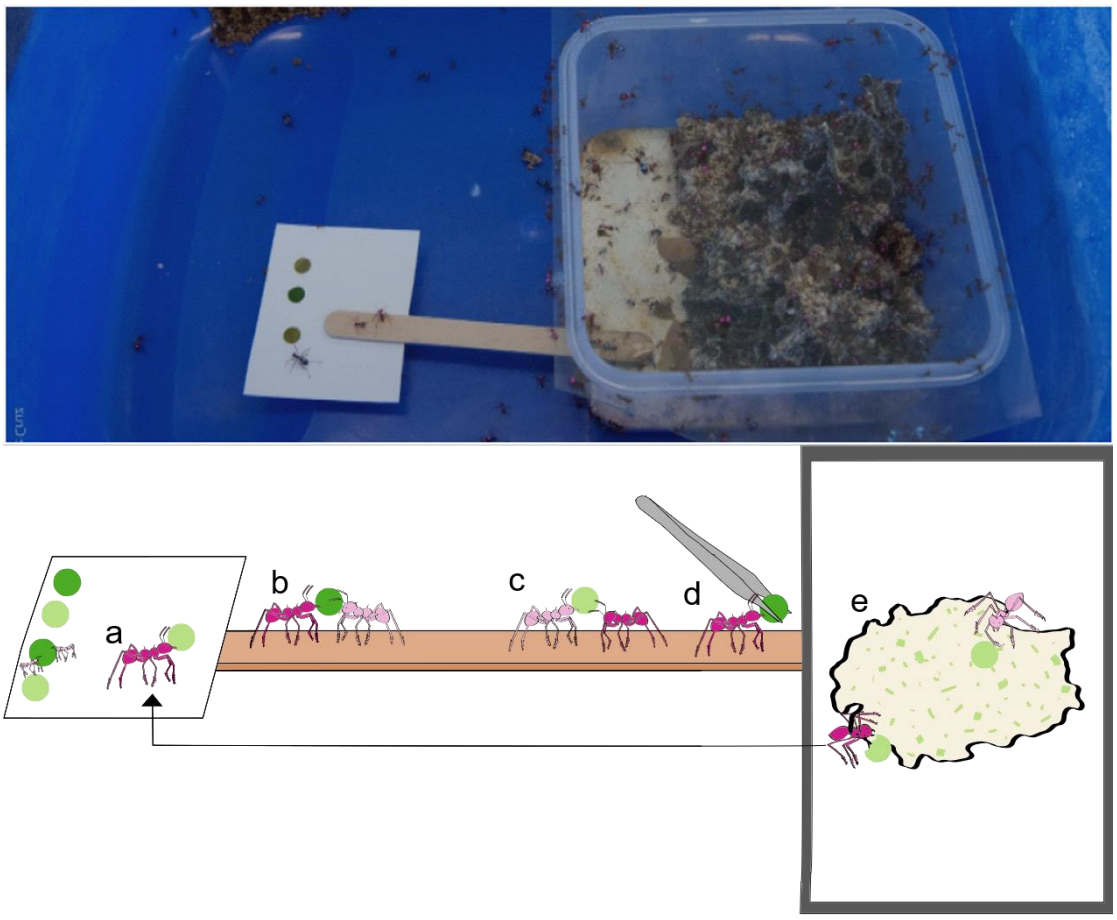

Figure 2.2 How might leaf rejection information be passed from experienced to naïve ants? The top panel is a photograph of the experimental set up. The bottom panel is an illustration of the same experimental set up depicting the hypotheses we tested regarding how naïve ants may learn about leaf quality from experienced ants. Naïve ants are shown in red, experienced ants are pink. Leaves of the previously treated type are dark green, and the never-treated leaf type is light green. Positive feedback mechanisms tested: A) Naïve ants prefer leaves that they have experienced incorporated in the garden. B) Naïve ants prefer leaves they encounter on the foraging trail carried by other ants. Negative feedback mechanisms tested: C) Minims block naïve ants from collecting some leaves. D) Naïve ants receive information about the leaf type they are carrying back to the nest via antennation from experienced ants. E) Experimentally removing carried leaves with forceps discourages ants from collecting them again.

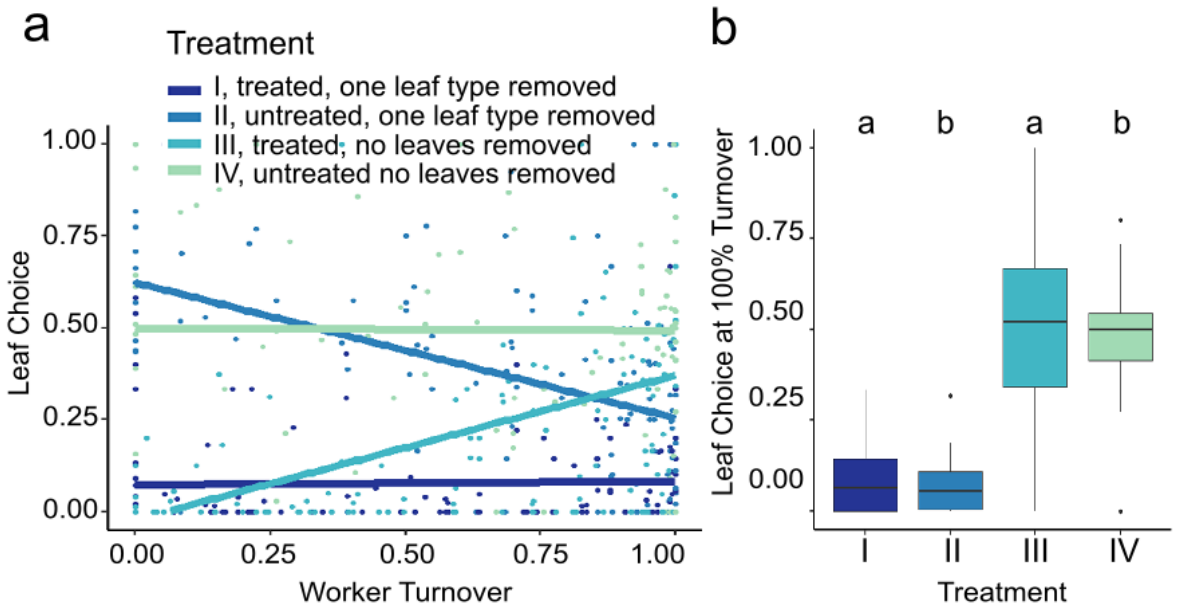

Figure 2.3 The proportion of leaves chosen by foragers that were of the previously treated type (or the untreated leaf that was offered on Day 0). **a)** Each line represents a linear approximation of the relationship between leaf choice and worker turnover for the four treatment groups. We have no biological reason to fit a linear function instead of a nonlinear function. Rather, we chose the simplest function and acknowledge that more work is needed to determine the precise relationship between the worker turnover process and collective memory of leaf choice in colonies, which may indeed be a nonlinear process. **b)** When all experienced workers had died and colonies reached 100% worker turnover, colonies' leaf choices no longer depended on whether or not they had received fungicide treatment on Day 0, but did depend on which leaves they were allowed to incorporate in their gardens throughout the experiment. In other words, the collective memory of leaf rejection died along with the last experienced workers. Letters in panel B indicate statistically significant differences between groups (GLMM effect of cycloheximide treatment when the proportion of naïve ants is 1.0, $z = 0.015$, $p = 0.99$, $df = 36$).

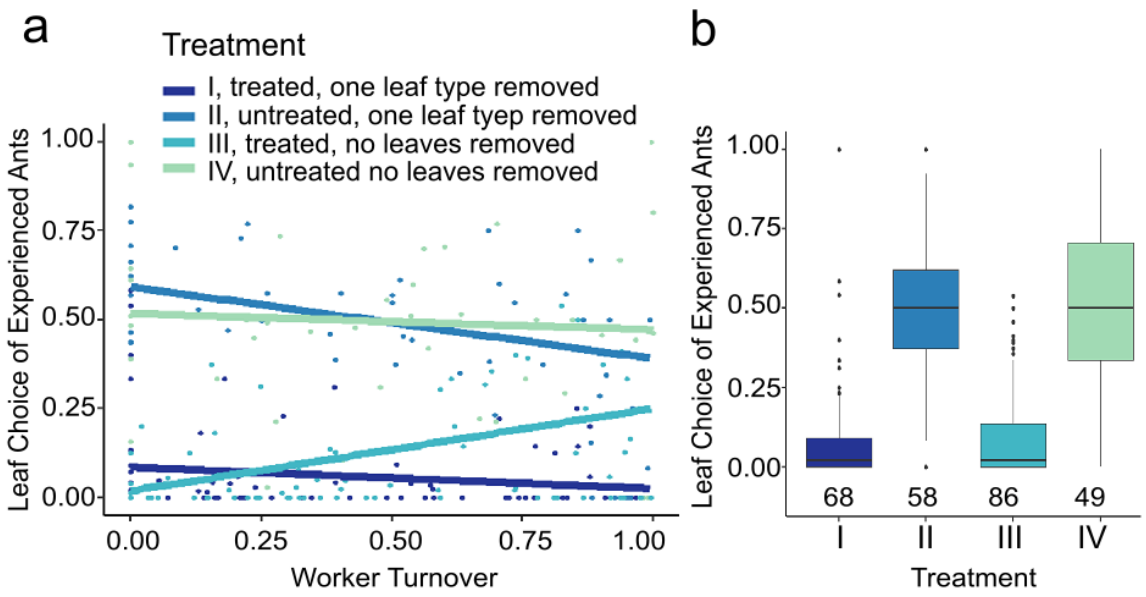

Figure 2.4 a) Time series of leaf choices by experienced ants. Each line represents a linear approximation of the relationship between leaf choice and worker turnover for the four treatment groups. **b)** Mean leaf choices for experienced ants in all colonies and all post-treatment timepoints for Treatments I-IV (See Table 2.1 and Fig. 2.1 for treatment details). Sample sizes are shown beneath each group. For each test leaf choice is measured as the proportion of collected leaf discs that are of the previously treated type. The leaf choices of experienced ants depend on their experience (or lack thereof) with fungicide treatment, but not by the pattern of leaf removal they experienced throughout the experiment (Statistical details in Table 2.3).

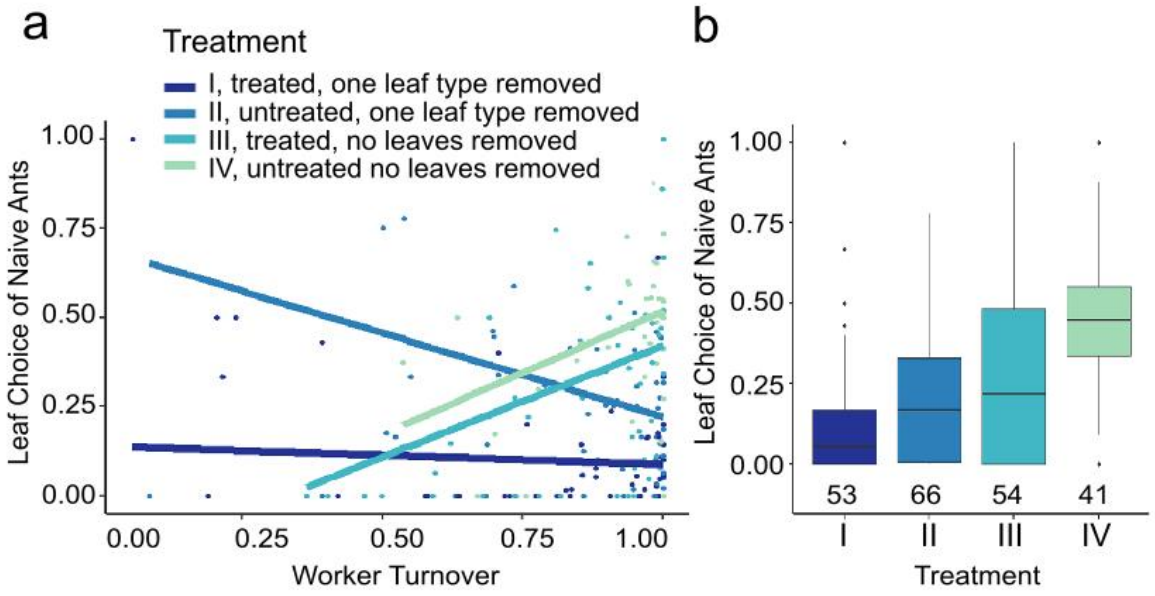

Figure 2.5 a) Time series of leaf choices by naïve ants. Each line represents a linear approximation of the relationship between leaf choice and worker turnover for the four treatment groups. **b)** Mean leaf choices for naïve ants in all colonies and all post-treatment timepoints for Treatments I-IV (See Table 2.1 and Fig. 2.1 for treatment details). Sample sizes are shown beneath each group. For each test, leaf choice is measured as the proportion of collected leaf discs that are of the previously treated type. The leaf choices of naïve ants depend on their nestmates' experience (or lack thereof) with fungicide treatment, and on the pattern of leaf removal they experienced throughout the experiment (additional statistical details are in Table 2.3).

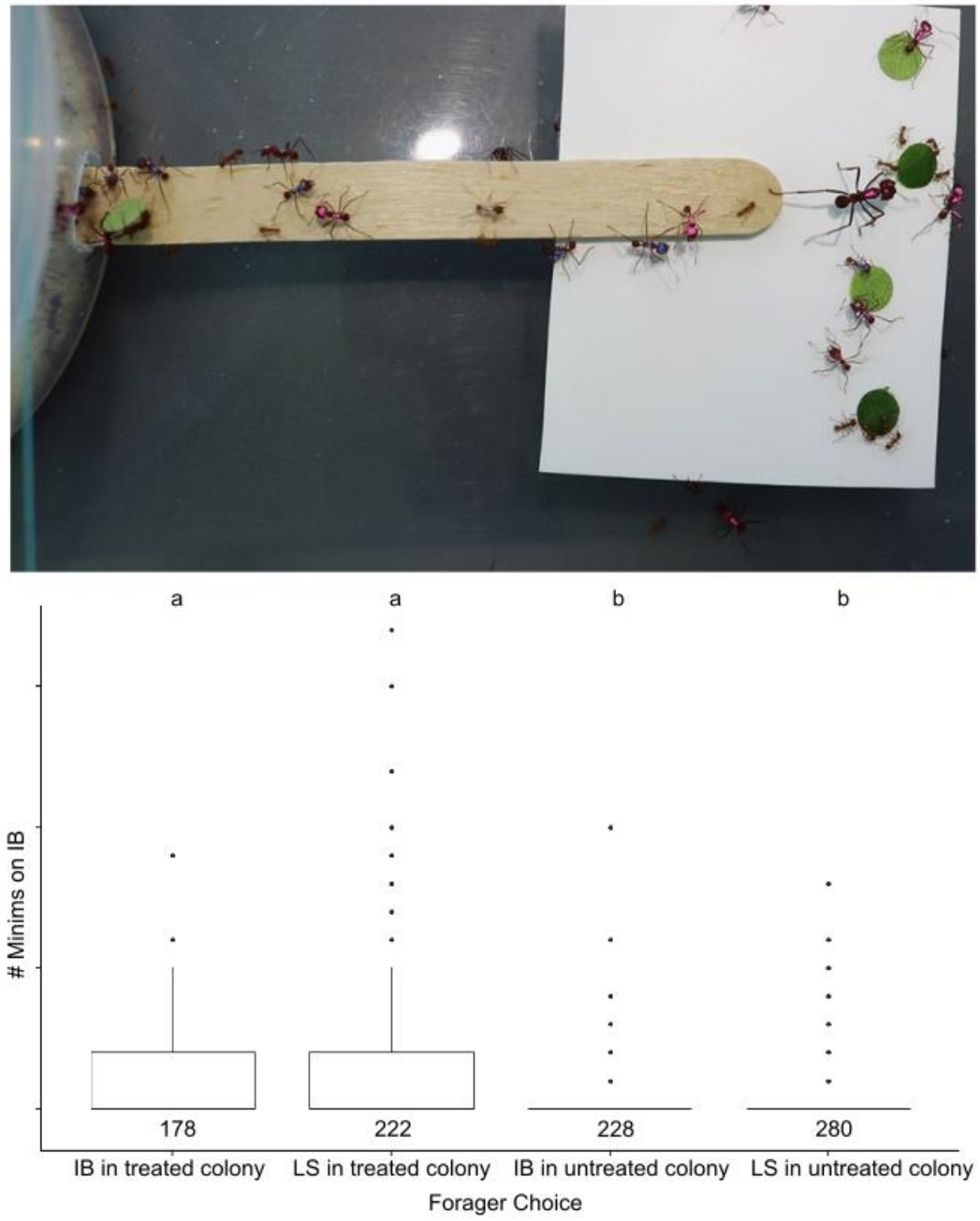

Figure 2.6 The top panel shows a photo of a leaf preference test of a cycloheximide treated colony from Treatment I. There are four minims on each of two dark green *I. batata* (IB) discs. This colony had received cycloheximide treated IB discs on Day 0. The bottom panel shows the numbers of minims on IB leaf discs for each of the four possible leaf choice scenarios. Letters in the bottom panel indicate statistically significant differences between groups. These analyses were done only for Treatments I and II. We counted the numbers of minims on IB leaf discs at the time foragers picked up a leaf. Numbers of foragers sampled for each leaf type in treated and untreated colonies are shown under each boxplot.

Table 2.1 Details of Treatments varying in 1) cycloheximide induced leaf rejection, and 2) type of leaf offered on Day 0 and, 3) possibility for updating leaf quality information based on leaf removal pattern.

	Treatments			
Treatment	Cycloheximide	Leaf type offered on Day 0 (with or without cycloheximide)	Leaf Removal Pattern	Number of colonies
I	Yes	*I. batata*	*L. speciosa* incorporated; *I. batata* removed	10
II	No	*I. batata*	*L. speciosa* incorporated; *I. batata* removed	11
III	Yes	*I. batata* (5) or *L. speciosa* (6)	Both leaf types incorporated	11
IV	No	*I. batata* (5) or *L. speciosa* (4)	Both leaf types incorporated	9

Table 2.2 Hypotheses, predictions, and results regarding how naïve ants may learn their leaf preferences from experienced ants. The data used are correlational, however, the hypotheses make distinct predictions. For each of these analyses we collected data from our existing leaf preference videos.

Hypothesis	Feedback Type	Prediction	Result	Statistical details
Ants collect the types of leaves they have detected in the garden	Positive	Relative leaf preferences of naïve ants will correlate with relative amounts of leaves that have been incorporated into the garden	Supported	See 'Past Leaf Incorporation' in Table 2.3
Naïve ants prefer leaves they encounter on the foraging trail carried by other ants	Positive	Naïve ants will be more likely to choose an *I. batata* leaf if they came into contact with more ant carrying *I, batata* leaves than *L. speciosa* leaves.	Supported	See 'Outbound Antennations' in Table 2.4
Minims block naïve ants from collecting some leaves	Negative	Naïve ants are more likely to pick up leaves that have fewer minims on them.	Rejected	See "Number of minims' in Table 2.4
Naïve ants are discouraged by nestmates when they carry home the "wrong" type of leaf	Negative	Ants carrying leaves of the previously treated type in treated colonies will receive more antennations on their inbound journey than ants in other contexts	Rejected	See 'Inbound antennations' in Results
Experimentally removing carried leaves with forceps changes the preference of ants that carried them	Negative	By age cohort, ants will become less likely to pick up *I. batata* leaves as the total number they have attempted over time increases.	Rejected	See 'Previous attempts collecting IB' Table 2.4

Table 2.3 Statistical results from 4 GLMM analyses testing for effects of cycloheximide treatment (i.e., initial leaf quality), leaf removal, and past leaf incorporation in experienced and naïve ants separately. We used the following command from the R package 'lme4' to test for effects of cycloheximide treatment and leaf removal pattern on the leaf choices of experienced or naïve ants: glmer.nb(PT_taken~ Treatment+ Leaf_removal+ offset(ln_LeavesTaken)+ (1|Week) + (1| Colony)+(1|Queen) + (1| Experimental_leaf), data = filter(nodouble_glmm, Agegroup=="old")). To test the effect of past leaf incorporation we used: glmer.nb(PT_taken~ Treatment_b+ PT_incorp_ever+ offset(ln_LeavesTaken)+ (1|Week)+ (1| Colony) + (1| Experimental_leaf), data = filter(cumsum_glmm, Age=="new")).

Age group	Cycloheximide treatment		Leaf removal pattern		Past leaf incorporation		Median, Mean Prev. Treated discs taken	Treatments included
	z	p	z	p	z	p		
Experienced ants	9.29	<0.001	0.46	0.649	-	-	2, 11.26	I-IV
Naïve ants	4.32	<0.001	7.00	<0.001	-	-	4, 11.87	I-IV
Experienced ants	4.25	<0.001	-	-	0.09	0.929	2, 8.85	III, IV
Naïve ants	0.42	0.678	-	-	2.68	0.007	15, 20.48	III, IV

Table 2.4 Statistical results from one mixed-effects, logistic regression model testing three hypotheses concerning the mechanisms leading to leaf preference in foraging ants. This model included data from Treatments I and II, but not III and IV. We used the following command from the R package 'lme4' to test whether any of our three mechanistic hypotheses lead to an ant's leaf choice (*I. batata* or *L. speciosa*): glmer(Choice_binary ~ TouchIBtiny+ Outbound_propIB + Prev_IB_attempts + (1|Week)+ (1| Colony) , data = data.full, family=binomial)

Hypothesis	Z	p	Treatments included
Outbound antennations with laden foragers [F_{IB}/ (F_{IB}+ F_{LS})]	2.21	0.0270	I, II
Number of minims on *I. batata* leaf discs at moment of choice	-0.79	0.4286	I, II
Previous (unsuccessful) attempts at collecting *I. batata* made by ants in this cohort	0.42	0.6756	I, II

Table 2.5 Statistical results from GLMM analyses testing for effects of cycloheximide treatment and leaf removal on leaf preferences of experienced and naïve ants, when naïve ants make up between 80 and 99% of their colonies. We used the following command from the R package 'lme4': glmer.nb(PT_taken~ Treatment+ Leaf_removal+ offset(ln_LeavesTaken)+ (1|Week) + (1| Colony)+(1|Queen) + (1| Experimental_leaf), data = filter(turnover80_99_glmm, Agegroup=="new")).

Age group	Cycloheximide treatment		Leaf removal pattern		Median, Mean Prev. Treated discs taken	Treatments included
	z	p	z	p		
Experienced ants	3.41	<0.001	1.60	0.110	1, 2.66	I-IV
Naïve ants	4.28	<0.001	6.91	<0.001	6, 14.98	I-IV

Chapter 3: Only young, naïve ants copy: how demography drives collective memory maintenance in leafcutter ants

ABSTRACT

Collective systems often employ dedicated mechanisms to maintain and disseminate knowledge held by some or all members. In animal groups, the transmission of information between individuals is termed 'social learning', and individuals need to balance personal information (i.e., directly acquired) and social (i.e., transmitted) information when making decisions. Here we ask how leafcutter ants achieve this balance to allow for updates to group 'memory' and to avoid the loss of information held by an experienced minority. Our study was in the context of experimentally induced leaf rejection via fungicide treatment, and we manipulated how age and task group (i.e., body size) of individuals related to their information status (i.e., experience). We found that only ants who were naïve to critical information (which leaves had been treated with fungicide) responded to social information from minims (small garden workers). And, we found that young, naïve ants copied their nestmates and rejected leaves of the previously treated type, but only when old, experienced ants did the majority of foraging trips (leaf pickups). This perhaps implies that young, naïve ants copy their nestmates regardless of age or experience, but we only see a change in their behavior when the majority of their nestmates have different information than they do. Collective memory, in ant colonies, thus appears to be maintained in part due to differing propensities to copy by different demographic groups. This study highlights the importance, and perhaps general role, of older age-cohorts to disseminate and maintain crucial information, and of younger age-cohorts to learn and innovate using selective, imperfect copying.

INTRODUCTION

For many animals, learning by copying others can save time and effort. The benefits of social learning by copying have been demonstrated across the animal kingdom (reviewed by, Leadbeater and Chittka 2007; Reader and Biro 2010; Thornton and Clutton-Brock 2011). Animals copy others to learn what to eat (Galef and Giraldeau 2001), where to find food (von Frisch 1967), how to avoid predators (Griffin 2004), and who to mate with (White 2004).

However, relying too heavily on social information can be costly (Giraldeau et al. 2002). Theoretical models and empirical studies have shown that the costs and benefits of using social information depend, for example, on resource distribution and the social environment. Animals are often better off relying on personal over social information in environments that have more evenly dispersed resources (Donaldson-Matasci and Dornhaus 2012; Cook et al. 2013; l'Anson Price et al. 2019), that change quickly (Dechaume-Moncharmont et al. 2005; l'Anson Price et al. 2019; but see Rendell et al. 2010), or that have low resource diversity (Donaldson-Matasci and Dornhaus 2012; l'Anson Price et al. 2019). Some animals use social information more often when it has been reliable in the past (Dunlap and Stephens 2016), or when other personally acquired cues are unreliable (Jones et al. 2013). And, the benefit of using social information is often frequency-dependent (Barnard and Sibly 1981). Social learning is most beneficial when most individuals learn directly from the environment. As more individuals copy, shared information becomes less reliable as fewer individuals are directly sampling changing environmental conditions. In addition, new resources that are found will be quickly depleted if many individuals are relying on social information (Szymkowiak et al. 2016).

A high reliance on social information within a group or population could lead to catastrophic cascades of misinformation due to the positive feedback effects of 'jumping on the wrong bandwagon' (Rieucau and Giraldeau 2011). Such misinformation cascades make systems, like social animal groups using copying, less flexible and more difficult to update as the environment changes.

Modeling and empirical studies suggest that groups with low relatedness may reach an equilibrium ratio of 'producers' (individuals who sample their environment) to 'scroungers' (individuals who copy others) (Barnard and Sibly 1981), where the benefits of both strategies are equal, due to frequency dependence. In groups with higher relatedness, competition within the group is less impactful, and thus the cost of being copied is reduced (Hamilton 1964; Frank 2003). As a result, social information is more likely to be shared freely and 'intentionally' as social signals (Krebs and Dawkins 1984). Signalling reduces the costs of using social information and thus decreases the ratio of individuals sampling their environment directly to individuals copying others (Mathot and Giraldeau 2010). Social insect colonies often flexibly divide their labor between a small group of individuals sampling their environment (scouts), and a larger group of individuals (recruits), using social information to exploit resources found by scouts (Oettingen-Spielberg 1949; Lindauer 1952; Seeley 1983; Jacobus and Biesmeijer 2001).

Many types of signals have evolved to facilitate social information exchange among workers in social insect colonies in the context of foraging. To find the locations of high-quality resources or to discriminate between high and low quality resources, social insects may use chemical signals (e.g., pheromone trails; Czaczkes et al. 2015), tactile signals (e.g., vibrations on the substrate and on nestmates; Hunt and Richard 2013), and/or visual signals (e.g., visual tracking of leaders in flight; Nieh 2004).

Social insect colonies also have the advantage of being able to share information among overlapping age cohorts. When workers vary in age and experience, is information sharing a two-way-street with respect to worker age, experience level, and/or task group? Or do younger, less experienced individuals use or provide social information differently than their nestmates? Meerkats (Thornton and Malapert 2009), birds (Catchpole & Slater 1995), cetaceans (Whitehead and Rendell 2014), and humans (Demps et al. 2012) have all been found to rely on social information more heavily as juveniles than as adults. And in *Temnothorax* ants, older and more experienced ants are more likely to lead tandem runs during house-hunting, and are more efficient in doing so (Franklin et al. 2012).

Although social learning strategies (e.g. concerning 'who to copy' and 'when to copy') have been found in diverse taxa, (Gruter and Leadbeater 2014; Mesoudi et al. 2016), it remains unclear how these individual social learning strategies lead to collective patterns at the level of the colony or population (Aplin 2019). Here, we investigated who copies whom in *Atta colombica* leafcutter ant colonies, and how selective copying may affect collective outcomes.

Leafcutter ants collect leaves which they use as substrate to grow gardens of fungus in underground chambers. The ants and their fungus are obligate mutualists; the fungus metabolizes leaf material provided by the ants and the ants eat the mycelium of the fungus. Tropical leafcutter ants collect leaves from as many as 50-80% of the plants in their foraging range (Cherrett 1989) and bring them to their fungus gardens, which provide feedback to the ants on the quality of the leaves they have collected. Leafcutter ants also use social information from their nestmates to determine which leaves are beneficial for their garden. Foragers leaving a nest often decide which substrates to collect based on what their nestmates carry home on the foraging trail (Roces 1990, 1994; Farji-Brener et al. 2010).

This study focuses on the use of social information in the context of learned leaf rejection. Laboratory and field studies have shown that leafcutter ants learn to reject species of plants whose leaves are detrimental to fungus garden growth (Herz et al. 2008; Saverschek et al. 2010b). Rejection is based on cues that are produced by fungus gardens during the 2-3 days after harmful leaves have been incorporated into a garden (Herz et al. 2008). After this cue from the fungus garden has decayed (after three days), there are other ways that foragers learn to avoid a particular type of leaf and to prefer other types of leaves. For example, ants can learn leaf avoidance and preference from bits of rejected leaf material discarded by workers in the waste (Arenas and Roces 2018), from beneficial leaf material stored by workers in the fungus chamber (Arenas and Roces 2018), from brief interactions between outgoing and incoming workers on the foraging trail (Roces 1990, 1994; Farji-Brener et al. 2010), and possibly from interactions with minims that spend most of their time in the fungus garden, and thus may have the best information about which leaves are suitable for the fungus gardens' growth (Saverschek 2010).

Leafcutter ants are polymorphic in size (Wilson 1980). The smallest ants, so-called minims, are generally thought to mostly tend to the fungus garden and care for the brood (Oster and Wilson 1978; Wilson 1980). However, minims also walk on the foraging trail, but they do not carry leaves (Stradling 1978). Rather, minims play a larger role than non-minims in maintaining pheromone trails (Evison et al. 2008), and they are seen riding back to the nest or 'hitchhiking' on leaves carried by larger foragers. Such hitchhiking behavior likely provides two types of defense as minims clean leaves of contaminants, and protect foragers from parasitic phorid flies on their ride back to the nest (Vieira-Neto et al. 2006). Minims have also been observed aggregating on leaves that were once experimentally treated with a fungicide (Chapter 2).

Collective memory of leaf avoidance is retained in colonies even when there is no persistent cue available in the fungus garden or the waste, and when ants who had access to the original fungal rejection cue are in the minority of workers (Chapter 2). Specifically, naïve ants, which have not experienced the original fungus cue, displayed leaf rejection even when they made up 80-99% of their colonies (Chapter 2). However, when all the old, experienced ants had died, and when young, naïve ants were allowed to incorporate leaves that were previously (but no longer) harmful into their fungus gardens, they learned to re-accept this leaf type.

Such a collective memory system is thus able to retain the knowledge of an experienced minority, while also remaining flexible enough to update over longer time scales (i.e., seasonally). To accomplish this leafcutter ants may have 1) some means of discrimination that ants use to decide who to copy, or 2) a difference in propensity to copy based on age, or past experience. These two possible mechanisms highlight the difference between a model-dependent selective copying rule that specifies 'who to copy' (e.g., ants are most likely to copy older or more experienced individuals) vs. a state-dependent rule that specifies 'when to copy' (e.g., young or inexperienced individuals are most likely to copy).

We manipulated colony compositions by age, experience and task group (i.e., body size) so that we could test for either model-dependent, 'who to copy' rules, or state-dependent, 'when to copy' rules (See Table 3.1). We also tested whether the proportion of foraging work that was done by experienced ants impacted the leaf choices of foragers.

METHODS

Colony Collection and Maintenance in the Laboratory

We collected 20 *Atta colombica* colonies in Gamboa, Panamá in April-June 2019. The colonies we collected included a range of sizes, but most were incipient colonies with 1-3 nest entrances (Weber 1972) and none had more than 5 entrances (for details on colony compositions, see below). We kept queenright colonies in a laboratory with large windows and natural light conditions, temperatures fluctuated between 23-26°C. Colonies were housed in enclosed plastic nest boxes (9x13x5 cm^3). We poured a layer of plaster in the bottom of each nest box and moistened the plaster with water as needed to maintain garden chambers near 100% humidity. Nest boxes were placed in larger plastic boxes (25x30x15 cm^3) with walls lined with Insect-a-slip (Bioquip, Rancho Dominguez, CA, USA) to prevent escape.

Colonies were fed a diet of organic oatmeal flakes and leaves from an *Adenanthera pavonina* tree (Fabales, Fabaceae) growing in Gamboa, Panamá. We chose *A. pavovina* as a primary food source because leaf cutter ants harvest from this tree naturally and do well when fed these leaves in the lab. When we started experiments, colonies ranged in size from 128-1189 workers, not including the smallest workers (hereafter referred to as 'minims', head width < 1mm). All colonies had one queen though not always the original queen. When requeening was required for a particular replicate colony, we removed a queen from her previous colony and carefully took off any bits of fungus or workers that clung her. We kept such queens isolated in plastic containers for about at least hour before using forceps to gently place them on the fungus garden of the recipient queenless colony. Requeening is generally thought to have no effect on foraging activity or leaf preference in *A, colombica* in the lab, but this remains to be tested. All colonies also contained all fungus and brood that were collected at the time of collection.

Painting Workers and Establishing Colonies in the Laboratory

After establishing colonies in the lab, we paint-marked all ants except minims (head width ~1mm or less). This first group of ants to be painted will be referred to as 'old foragers'. And, all ants large enough to be painted (head width > 1mm), will be referred to as foragers. Every 2-3 weeks we painted newly-eclosed ants a new color to distinguish different age cohorts of ants. Ants painted during this time are referred to as 'young' ants. Before colonies were treated with cycloheximide (see below), we fed them both experimental leaf types, *Hibiscus* and *Lagerstroemia speciosa*, in addition to substrates listed above. *H. hibiscus* leaves were collected from plants we grew outside our laboratory in pots. *L. speciosa* leaves were collected from multiple trees growing in the town of Gamboa. Both types of leaves were collected fresh every day. After we treated colonies with cycloheximide, we no longer fed them either of the experimental leaf types to ensure that ants could not access additional information regarding leaf quality from their fungus gardens. Figure 3.1 shows a diagram of the experimental schedule.

Splitting of Colonies to Generate Experimental Subcolonies

We split colonies into two subcolonies when we observed the 'young' age group ants participating in leaf collection. We noticed that young workers typically started to participate in foraging about 3-5 weeks after they eclosed and were painted. We then carefully divided the ants by age group into two separate, new nest chambers. Each subcolony was composed of half the old ants, half the young ants, and half of the fungus garden from their source colony. We did not control the ratios of young to old ants in the manipulated colonies, but we later tested for the effect of that variable in our models. Previous experiments found that a young cohort of *A. colombica* forages with an old cohort when the young ants comprise ~70-99% of their colony (Marti et al

2020). We assume all our colonies were near this range of age-composition, but we did not quantify the exact ratios of young to old ants in each colony. We removed the original queen and re-queened each subcolony with a foreign queen. These queens were taken from other *A. colombica* colonies we maintained in the lab. All re-queenings were successful, meaning ants began to clean the new queen immediately after she was introduced and did not attack her.

Cycloheximide Treatment

We used a hole punch to create leaf discs, 6 mm in diameter. We treated these leaf discs with a low concentration solution of cycloheximide (0.03% w/w) through suction infiltration (Herz et al. 2008). When ants collect cycloheximide-treated leaves and incorporate them into their fungus gardens, ants learn to reject leaves from that species of plant, and then reject leaves even when no fungicide is present in future trials (Herz et al. 2008; Saverschek et al. 2010b; Saverschek and Roces 2011).

We pseudo-randomly allocated colonies to be treated with either *H. hibiscus* or *L. speciosa*. For each pair of subcolonies, we randomly chose one subcolony to receive 30 cycloheximide-treated leaf discs, while the other subcolony received 30 untreated discs of the same type of leaf.

Swapping Ants Between Treated and Untreated Subcolonies

Ants learn leaf rejection from their fungus gardens in the first 2-3 days following incorporation of a harmful leaf (Herz et al. 2008), but not after that time period. We therefore waited seven days so that any leaf rejection cues from the fungus garden would no longer be detectable by the ants. We also removed all waste from each nest box so that rejection information could not be learned from the waste (Arenas and Roces 2018). Once at least seven days had passed

since the fungicide treatment and all waste was removed, we swapped either the young or old cohort non-minims (head width > 1mm) between the treated and untreated subcolonies. The other age cohort (young or old) remained in the nest along with the queen, fungus garden, and minims. We separated out and swapped the old ants from half of the colonies that were treated with each leaf type. And we separated out and swapped the young ants from the other half of the colonies that were treated with each leaf type. After we performed the swaps, we had four different colony compositions (Fig 3.2). These four different colony compositions allowed us to isolate the effects of age, experience level, and task group (i.e., body size) on the use of social information. Old, experienced ants were always paired in mixed colonies with young, naïve ants. Old, naïve ants were always paired with young, experienced ants. And, we also included each of these combinations with either experienced or naïve minims (Fig 3.2). Swapped subcolonies were set up in the experimental arenas shown on the far right in Figure 3.1.

8-Hour Foraging Tests

We set up colonies in an experimental arena (Fig 3.1, right). Each arena contained a plastic nest chamber, lined with plaster to retain high humidity, an extraction chamber, and 23.5cm long foraging trail and a 5x6 cm^2 foraging platform. We placed two discs of *H. hibiscus* and two discs of *L. speciosa* on the foraging platform and replaced discs as ants collected them. We always took leaf discs away from foragers when they reached the extraction vestibule that was placed between the foraging trail and the nest entrance. This prevented ants from gaining additional information regarding leaf quality from their fungus gardens, which might let them know that both leaves were harmless. We recorded all ants' leaf choices over the course of the 8-hour test. We divided each

8-hour test into two 4-hour time blocks in order to compare ants' choices between the first and second halves of the day.

Experimental Design

We designed this experiment to test five hypotheses regarding selective copying in leafcutter ant colonies (see Table 3.1 for hypotheses and predictions). We made experimental subcolonies to test for copying between old, experienced foragers and young, naïve foragers, and between old, naïve foragers and young, experienced foragers. We also manipulated the experience of minims to test whether foragers of any age or experience level copy minims. When we swapped foragers between subcolonies; minims, the fungus garden, and the queen all stayed together as a unit after subcolonies were split and treated. This means that the variable 'minim experience' also includes fungus gardens and queens who did or did not experience the cycloheximide treatment. We think it is most likely that any effect coming from this variable is due to minims, not the queen or the fungus. A study on *Acromyrmex lundi* found that leaf rejection cues from the fungus garden only affect foragers' leaf choices for the first 2-3 after cycloheximide treatment (Herz et al. 2008). But, we cannot definitively rule out the queen or fungus as possible sources of leaf rejection information.

We also tested whether the proportion of work done by experienced ants influenced the leaf preferences of any group of foragers. This proportion refers to the number of leaf choices made by experienced ants divided by the total number of choices made by experienced and naïve ants during a given 4-hour time block. We did not manipulate this variable, but found a lot of variance across its range.

Statistical Methods

We used the following command from the 'lme4' package to generate the glmm which tested for the effects of different potential sources of social information on the leaf choices of experienced and naïve ants: glmer.nb(PT_taken ~ Forager.exp + Forager.age + Minim.exp + Time.block+Treated.leaf+Forager.exp*Forager.age+Prop_For_Exp+offset(ln_LeavesTaken)+(1| Colony),data=exp_naïve). The response variable (PT_taken) is the number of leaves of the previously treated type taken by ants divided by the total number of leaves taken of both types. For each mixed subcolony we measured naïve and experienced ants' preferences separately, each during two, four-hour time blocks, the first half of the day and the second half of the day. This means that for each subcolony there are 4 data points. With 40 subcolonies, we had 160 data points in total. We included forager age, forager experience, interaction between forager age and forager experience, minim experience, time block (first or second half of the day), the type of leaf that was treated with cycloheximide, and the proportion of work done by experienced ants as fixed effects (See Table 3.1 for predictions).The proportion of work done by experienced ants was measured as the number of leaf choices made by experienced vs. naïve ants. For a given trial, age and experience are linked, we know age based on paint markings and experience based on colony ID. As such, we were able to separately calculate the proportion of leaf choices for each of the four types of foragers. We included colony ID as a random effect.

We also used similar GLMMs for datasets including only experienced foragers and only naïve foragers. For these models we tested whether the proportion of leaves of the previously treated type taken by naïve or experienced ants could be explained by forager age, minim experience, time block (first or second half of the day), the type of leaf that was previously treated with cycloheximide, the proportion of work done by experienced ants, and the interaction between

forager age and the proportion of work done by experienced ants (See Table 3.1 for predictions). We included colony ID as a random effect. We used the 'lme4' package to generate GLMMs (Bates et al. 2015). We used the 'emmeans' function to report effect sizes of each significant fixed effect from the 'emmeans' package (Lenth 2020). All statistical analyses were performed in R 3.6.1 (R Core Team, 2019).

RESULTS

Do foragers copy when young?

We found that young ants do not always copy old ants (Fig 3.3). If this were true 1) young, naïve ants foraging with old, experienced ants would have copied them and rejected leaves of the previously treated type, 2) young, experienced ants foraging with old, naïve ants would have copied them and accepted leaves of the previously treated type, 3) old, naïve ants would not have copied, and would have accepted leaves of the previously treated type, and 4) old, experienced ants not have copied, and would have rejected leaves of the previously treated type. In the model described in Table 3.3, we would have thus seen a significant interaction effect between forager age and forager experience on leaf choices of experienced and naïve ants. This was not the case (forager age * forager experience: $z = -0.265$, $p = 0.791$, Table 3.3; Fig 3.3).

Do foragers copy when naïve?

We found that naïve ants do not always copy experienced ants (Fig 3.3). If they did, we should have seen each group of foragers reject leaves of the previously treated type, because every experimental colony contained one group of experienced foragers (i.e., there should have been no effect of forager age or forager experience on leaf choice). There was indeed no effect of forager age on leaf choice, but there was an effect of forager experience on leaf choice, with experienced

ants collecting fewer leaves of the previously treated type than naïve ants (forager age: $z = 0.388$, $p = 0.698$; forager experience: $z = 5.939$, $p < 0.001$; Table 3.3). On average, naïve ants collected an estimated 2.4 times more (naïve: 45.0 vs. experienced: 18.5) leaf discs of the previously treated type than experienced ants.

Neither young, nor old, experienced ants copied their nestmates (Fig 3.3, Fig 3.5). There was no effect of forager age ($z = -0.927$, $p = 0.354$; Table 3.4; Fig 3.3), the proportion of work done by experienced ants ($z = -0.815$, $p = 0.415$; Table 3.4; Fig 3.5), or minim experience ($z = 1.7$, $p = 0.510$; Table 3.4; Fig 3.4) on the leaf choice of experienced ants.

Do foragers copy when young and naïve?

We found that young, naïve ants matched the leaf preferences of the majority of their nestmates (Fig 3.5). When young, naïve ants did the majority of the foraging work they accepted leaves of the previously treated type. But, when their old, experienced nestmates did the majority of the foraging work they rejected leaves of the previously treated type. Specifically, young, naïve ants collected an estimated 2.2 times fewer discs of the previously treated type when old, experienced ants did the majority of work (14.3 discs at max = 99.5%), compared to when old, experienced ants did no work (31.4 discs at min = 0%). See Table 3.5 for details (forager age * proportion of work done by experienced ants: $z = -2.88$, $p = 0.004$; Fig 3.5).

Old, naïve ants did not match the leaf preferences of the majority of their nestmates (Fig 3.5). In fact, old, naïve ants collected an estimated 3.8 times *more* discs of the previously treated type when young, experienced ants did the majority of work (44.7 discs at max = 99.5%), compared to when young, experienced ants did no work (11.7 discs at min = 0%). See Table 3.5

for details (forager age * proportion of work done by experienced ants: $z = -2.88$, $p = 0.004$; Fig 3.5).

Does the minority copy the majority?

We found that, in general, the group of ants doing the minority of work (as measured by the number of leaf choices) does not copy the group of ants doing the majority of work (Fig 3.6). If this were true, we would expect that all ants would reject leaves of the previously treated type when experienced ants did the majority of work. And, we would expect that all ants would accept leaves of the previously treated type when naïve ants did the majority of the work. In the model including experienced and naïve ants' choices, there was no effect of the proportion of work done by experienced ants on leaf choice (proportion of work done by experienced ants: $z = 0.650$, $p = 0.515$; Table 3.3; Fig 3.6).

Do foragers copy minims?

We found that foragers do not always copy minims (Table 3.3). If they did, we would expect that each group of foragers in trials with experienced minims would reject leaves of the previously treated type, while each group of foragers with naïve minims would accept leaves of the previously treated type. In the model described in Table 3.3, we would have seen a significant effect of minim experience on leaf choice. There was however no effect of minim experience on leaf choice in the full model (minim experience: $z = 1.136$, $p = 0.256$; Table 3.3). But when we separated our data by forager experience, we found that there was an effect of minim experience on the leaf choices of naïve ants. We found that naïve ants collected an estimated 1.18 times fewer (naïve foragers with experienced minims: 46.3 vs naïve foragers with naïve minims: 54.6) leaf

discs of the previously treated type in the presence of experienced (vs. naïve) minims (experience of minims: $z = 2.038$, $p = 0.042$; Table 3.5; Fig 3.4). We found that the experience of minims had no impact on the leaf choices of experienced foragers (experience of minims: $z = 0.659$, $p = 0.510$; Table 3.4; Fig 3.4). The mechanism of this information transfer remains unclear.

DISCUSSION

We studied how ants of different ages, experience levels, and task groups use and/or provide social information to nestmates in a way that maintains collective memory of leaf rejection. We found that ants copied the most when they were both young and naïve. Ants that experienced fungicide treatment themselves responded very little to social information. Rather, experienced ants relied almost entirely on their own memories, that is, they maintained leaf rejection behavior in all social contexts. Ants who had never experienced fungicide treatment in their garden (naïve ants) responded differently to social information based on their age. Notably, the proportion of work done by experienced ants, and thus probably the encounter rate with such ants, had a large impact on the leaf choices of their naïve nestmates. But, the direction of this relationship depended on the age of naïve ants. Young, naïve ants matched the leaf preferences of the majority of their nestmates. Old, naïve ants on the other hand, displayed the opposite leaf preferences of the majority of their nestmates. We do not know why this would be. But, other studies have found that social insects can adjust their foraging behavior to balance colony-level intake of different nutrients, such as protein and carbohydrates (Dussutour and Simpson 2009) and essential amino acids (Hendriksma and Shafir 2016; Shik et al. 2016).

Leafcutter ants are known to use positive feedback mechanisms to determine which leaves are beneficial for their garden. Foragers leaving a nest often decide which substrates to collect

based on what they detect their nestmates carry home on the foraging trail (Roces 1990, 1994; Farji-Brener et al. 2010). Here, we see that this is true for naïve ants, but not for ants that experienced fungicide treated leaves in their gardens. It remains unclear what mechanisms foragers use to learn from minims, or whether this information might be coming from the queen or garden, rather than the minims per se. But, because minims are not large enough to carry the leaf discs we provided, we know that foragers must use a different mechanism to learn from minims vs. other foragers.

In this study, ants faced a tension between conflicting personal and social information. While 'naïve ants' in this study were relatively less experienced than our 'experienced ants', they were not truly naïve to the quality of the two experimental leaf types. Rather, naïve ants had positive experiences with both leaf types in their fungus gardens before the experiment began. Here, we saw that the tension between conflicting personal and social information was resolved differently based on ants' age and previous experience. Experienced ants tended to prioritize personal information over conflicting social information from naïve foragers. And, naïve ants needed also to be young in order to prioritize social information from experienced foragers over their own conflicting personal information.

Other animals also use context-dependent rules when faced with conflicting personal and social information about where to forage (reviewed by Kendal et al. 2018). For example, guppies and minnows will use conflicting social information when the costs of using personal information are high (e.g., predation risk), but not when the costs of using personal information are low (Kendal et al. 2004; Webster and Laland 2008). And, bison will use social information over conflicting personal information when they are more familiar with their groupmates (Merkle et al. 2015).

In this context, leafcutter ant foragers are most likely using a state-dependent strategy, 'when to copy' social learning strategy, rather than a model-dependent 'who to copy' strategy. Ants that are young and naïve use social information consistently, meanwhile, no one type of forager (by age, experience or task group) is consistently copied by others. Other animals are also known to use state-dependent social learning strategies based on age (Catchpole and Slater 1995; Thornton and Malapert 2009; Whitehead and Rendell 2014) or on stress levels (Lindeyer et al. 2013).

In this study, copying by young, naïve ants is also frequency-dependent. Young, naïve ants matched the leaf preferences of whichever group of foragers was doing the majority of the foraging work. As such, it appears that young, naïve ants may always use social information. But, a change in their behavior is only evident when they are frequently contacting experienced ants on the trail, who have different leaf preferences than they do.

Our study is one of few to determine how individual social learning strategies lead to group-level patterns, outside of primates. In humans, the social learning strategy 'copy the majority' leads to the spread of popular traits and the loss of rare traits, at the population level (Morgan et al. 2012). Also in humans, it is possible that copying is dependent on emotional-charged content leads to the spread of 'fake news' across the internet (Vosoughi et al. 2018). And in chimpanzees, the social learning strategy 'copy dominant individuals' leads to relative cultural stability in groups and cultural diversity across groups, despite high rate of migration between groups and innovation by young individuals within groups (Kendal et al. 2015).

Aplin (2019) compares cultural evolution to biological evolution in that social learning strategies allow beneficial solutions to spread through the group or population, and in that copying

errors introduce variation as do mutations. We found that leafcutter ants use a state-dependent social learning rule, ‘copy when young and naïve’, to allow leaf rejection behavior to spread through colonies. When this behavioral rule exists in the context of the constant, gradual turnover of individuals in a colony, and other age-based differences in behavior, the emerging colony-level system is both flexible and capable of retaining information.

Table 3.1 Five hypotheses tested along with corresponding predictions and results. For each hypothesis, the response variable is the number of leaves of the previously treated type divided by the total number of leaves of both types that were taken by old or young foragers, during the first or second time block. ***Denotes significance at $p < 0.05$.

Hypothesis	Qualitative Prediction: Who will accept or reject leaves of the previously treated type?	Quantitative Prediction: significant effect of [...] on response variable	Result	Outcome & Comparison to other systems
Copy based on age	Foragers will reject leaves of the previously treated type when experienced foragers are old.	Significant effect of *Forager age* * *Forager experience*	*Forager age* * *Forager experience*: p = 0.791, **NS** Table 3.3 & Fig 3.3	**Rejected** age-based copying has been seen in: birds (Catchpole and Slater 1995); cetaceans (Whitehead and Rendell 2014); & humans (Demps et al. 2012)
Copy based on experience	All groups will reject because all subcolonies contain one group of experienced ants.	No effect of either *Forager age* or *Forager experience*	*Forager age*: p = 0.698, **NS** *Forager experience*: p < 0.001, ******* Table 3.3 & Fig 3.3	**Rejected** experience-based copying has been seen in: humans (Wood et al. 2013); chimpanzees (Kendal et al. 2015)
Copy based on age and experience	Only young, naïve ants will copy and thus reject with old, experienced ants. All other groups will not copy.	For experienced ants, no effect of *Forager age* For naïve ants, significant effect of *Forager age*	For experienced ants, *Forager age*: p = 0.354, **NS** Table 3.4 & Fig 3.5	**Supported** young copy when naïve to stress: quail (Boogert et al. 2013)

			For naïve ants, *Forager age*: p = 0.004, ******* Table 3.5 & Fig 3.5	
Copy the majority, regardless of age or experience	All foragers reject when proportion of work done by experienced ants is high. All foragers accept when proportion of work done by experienced ants is low.	Significant effect of *Proportion of work done by experienced ants*	Across all ants, *Proportion of work done by experienced ants*: p = 0.515, **NS** For naïve ants, *Proportion of work done by experienced ants* * *Forager age*: p = 0.004, ******* Table 3.3, Table 3.5 & Fig 3.6	**Rejected** frequency-based copying has been seen in: Fish (Rendell et al. 2011); humans (Morgan et al. 2012); ants (Detrain and Deneubourg 2008)
Foragers copy minims, or copy based on task group	All foragers with experienced minims reject. All foragers with naïve minims accept.	Significant effect of *Minim experience*	*Minim experience* on leaf choices of all ants: p = 0.256, **NS** *Minim experience* on leaf choices of only naïve ants: P = 0.042, ******* Table 3.3, Table 3.5, Fig 3.4	**Rejected**

Table 3.3 Results of GLMM analysis of full data set. We tested whether the variation in the number of leaves of the previously treated type taken by ants could be explained by forager experience, forager age, minim experience, the type of leaf that was treated, the time block, proportion of work done by experienced foragers, and interactions between forager age and forager experience. We estimated effect sizes using estimated marginal means in the 'emmeans' package.

Variable	Effect size on # leaves of the previously treated type (median = 28.5, mean=58.96)	z score	p value
Forager experience (Naïve vs. Experienced)	26.5	5.939	<0.001
Forager age (Young vs. Old)	NS	0.388	0.698
Minim experience (Naïve vs. Experienced)	NS	1.136	0.256
Type of leaf treated (LS vs. HH)	30.8	3.392	<0.001
Time block (First vs. Second)	NS	0.351	0.726
Proportion of work done by experienced ants	NS	0.650	0.515
Forager age * Forager experience	NS	-0.265	0.791

Table 3.4 Results of GLMM analysis of experienced-ant data set. We tested whether the variation in the number of leaves of the previously treated type taken by ants could be explained by the experience of foragers, forager age, the experience of minims, the type of leaf that was treated, the time block, the proportion of work done by experienced ants, and the interaction between forager age tand the proportion of work done by experienced ants. We estimated effect sizes using estimated marginal means in the 'emmeans' package.

Variable	Effect size on # leaves of the previously treated type (med = 13.0, mean=32.97)	z score	p value
Forager age (Young vs Old)	NS	-0.927	0.354
Minim experience (Naïve vs Experienced)	NS	0.659	0.510
Type of leaf treated (LS vs. HH)	19.81	3.168	0.002
Time block (First vs. Second)	NS	0.322	0.747
Proportion of work done by experienced ants	NS	-0.815	0.415
Interaction between forager age and the proportion of work done by experienced ants	NS	0.986	0.324

Table 3.5 Results of GLMM analysis of naïve-ant data set. We tested whether the variation in the number of leaves of the previously treated type taken by ants could be explained by the experience of foragers, forager age, the experience of minims, the type of leaf that was treated, the time block, the proportion of work done by experienced ants, and the interaction between forager age and the proportion of work done by experienced ants. We estimated effect sizes using estimated marginal means in the 'emmeans' package.

Variable	Effect size on # leaves of the previously treated type (med = 69.0, mean = 84.9)	z score	p value
Forager age (Young vs Old)	0.5	2.847	0.004
Minim experience (Naïve vs Experienced)	8.3	2.038	0.042
Type of leaf treated (LS vs. HH)	30.4	2.086	0.037
Time block (First vs. Second)	8.4	-2.048	0.041
Proportion of work done by experienced ants	6.1	3.088	0.002
Forager age * proportion of work done by experienced ants	33.0	-2.888	0.004

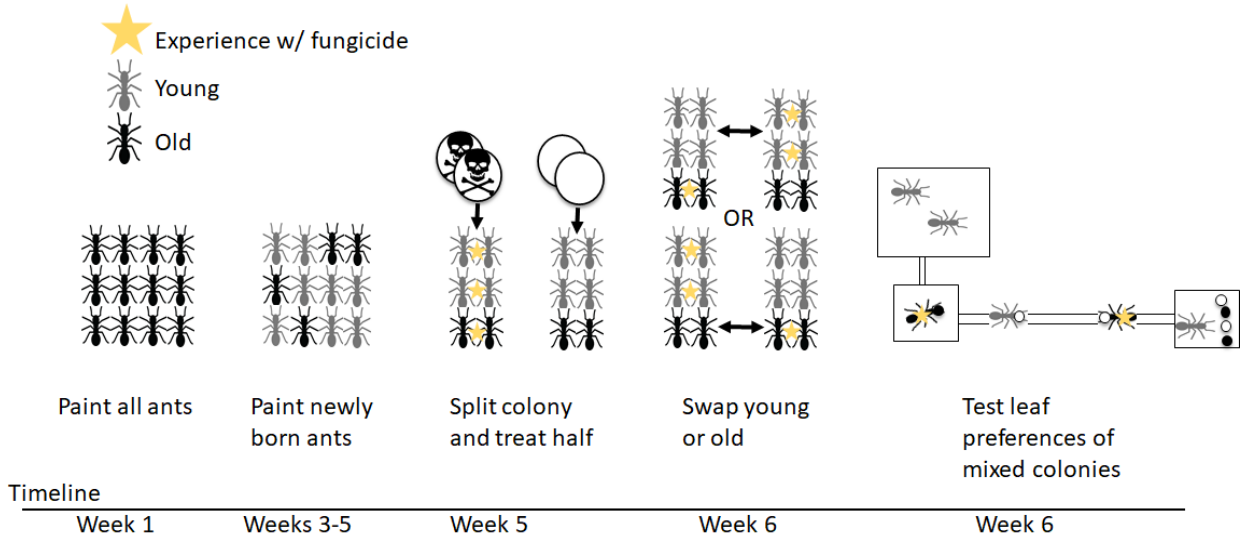

Figure 3.1 Diagram of experiment schedule. 1) We painted all ants in the colony the same color. 2) We waited 2-3 weeks for new ants to eclose and painted them a second color. 3) We split colonies in half, making sure that equal numbers of both age groups went to each half. Then, we gave one half of the colony either *H. hibiscus* or *L, speciosa* leaves treated with cycloheximide fungicide. We gave the other half of that colony the same type of leaf but without fungicide. 4) After at least one week, we swapped either the old or the young groups of ants between treated and untreated halves of the same colony. 5) We transferred ants to a test arena and tested their leaf preferences over the course of an 8-hour foraging day. Black ants are old, grey ants are young. Yellow stars represent experience with fungicide treatment. Skull and crossbones represent fungicide treatment on leaves. White circles are leaf discs of the previously treated type. Black circles are leaf discs of the type that was never treated.

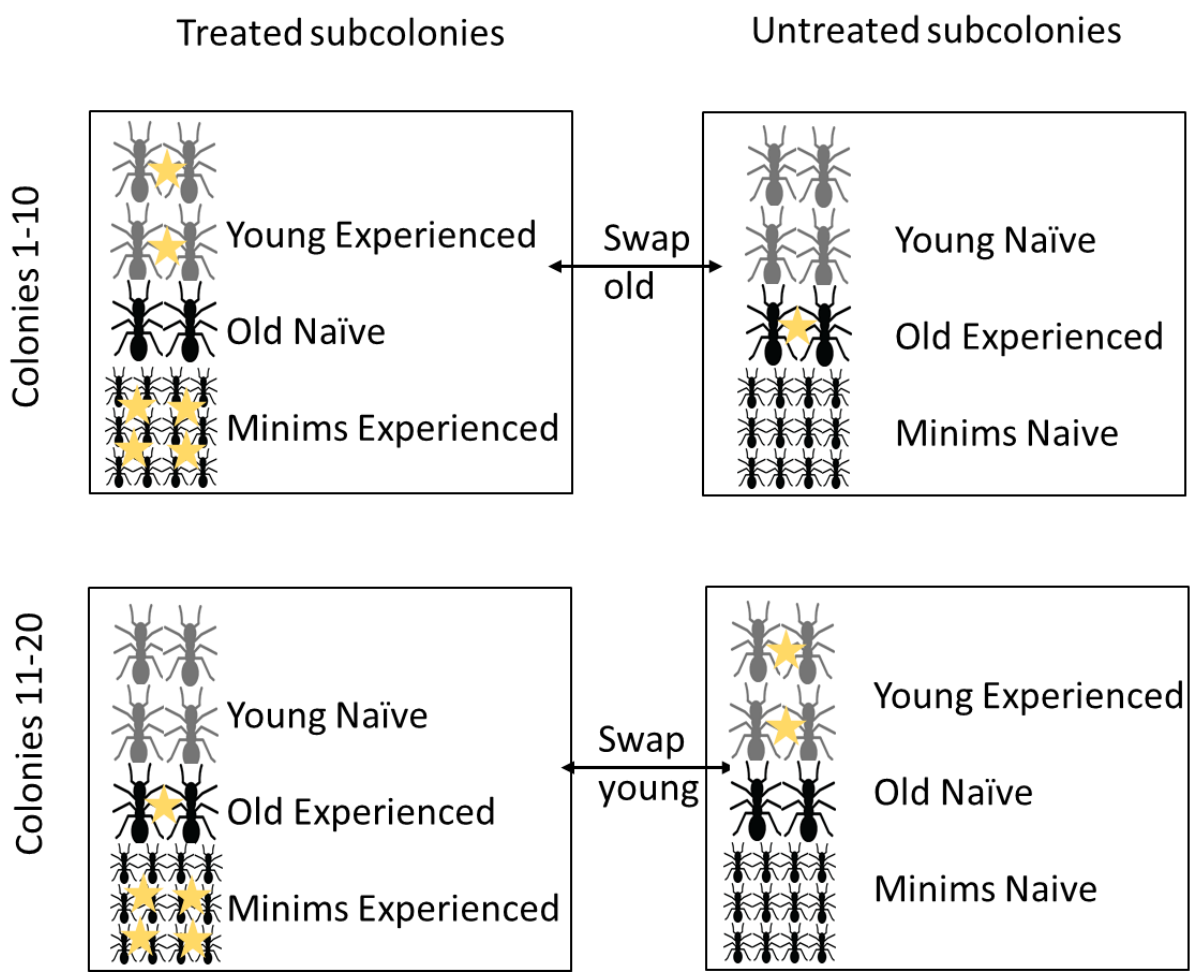

Figure 3.2 Diagram showing how two colonies were split and swapped to generate four social contexts. All subcolonies have new queens from other colonies. All subcolonies were rehoused in new nest boxes after we swapped young or old foragers. Fungus gardens and minims always stayed together, so any subcolony with experienced minims also had an experienced fungus garden. But, enough time (at least seven days) had passed that ants should no longer be able to detect leaf rejection cues from the fungus garden after the swap. Black ants are old, grey ants are young. Yellow stars represent experience with fungicide treatment.

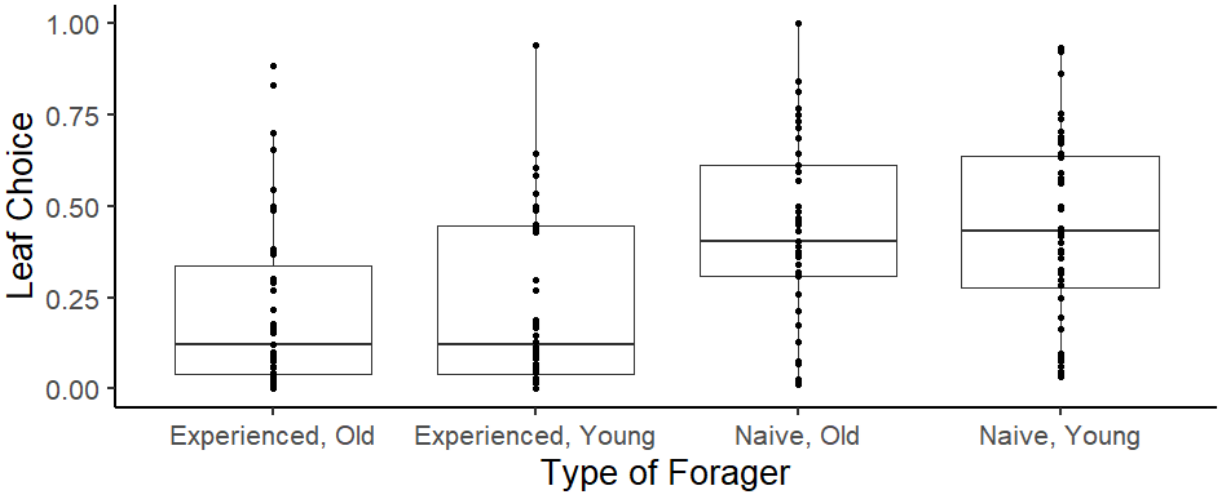

Figure 3.3 Experienced ants show decreased acceptance of leaves of the previously treated type compared to naïve ants (Forager experience: $z = 5.939$, $p < 0.001$). There was no effect of forager age on the leaf choices of experienced and naïve ants (Forager age: $z = 0.338$, $p = 0.698$). There was no significant interaction between forager experience and forager age (Forager experience * Forager age: $z = -0.265$, $p = 0.791$). For each of the 40 trials there are two data points for old ants and two data points for young ants. Each age group has a data point for the first 4-hour time block and the second 4-hour time block. For any given trial age and experience are linked. The total sample size is 160. See Table 3.3 for additional statistical details.

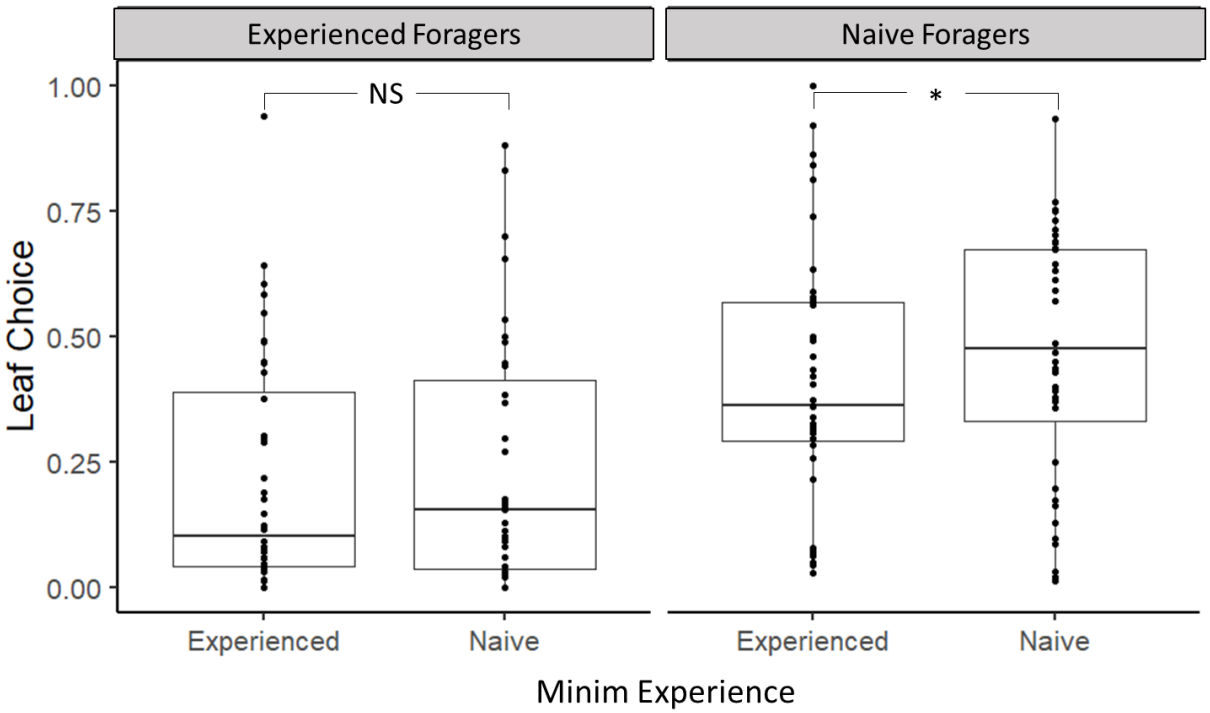

Figure 3.4 There is a small, but significant effect of minim experience on the leaf choices of naïve foragers, right panel (Minim experience: z = 2.038, p = 0.042; Table 3.5). There is no effect of minim experience on the leaf choices of experienced foragers, left panel (Minim experience: z = 0.659, p = 0.510; Table 3.4).

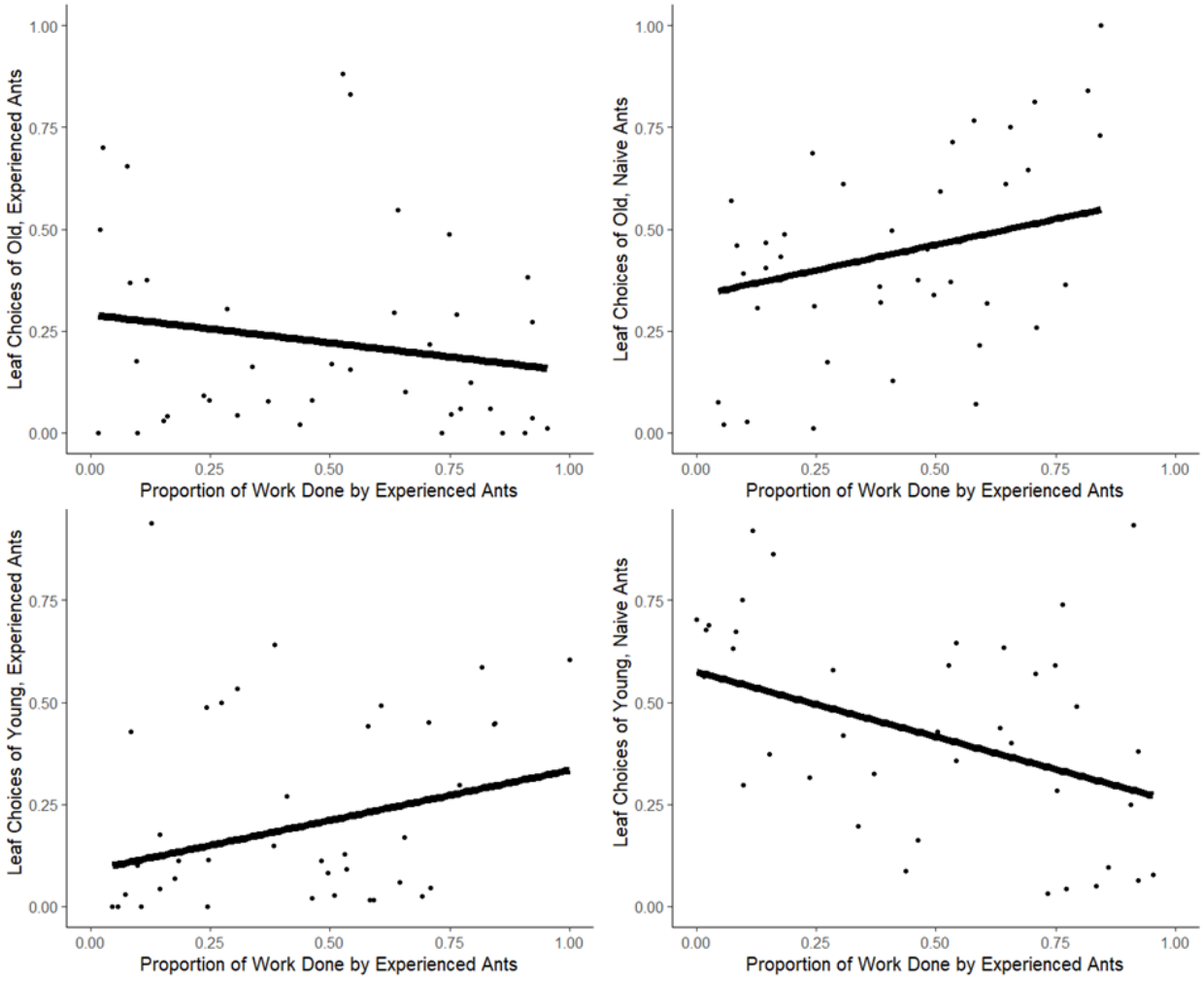

Figure 3.5 The proportion of work done by experienced ants has no effect on the leaf choices of experienced ants of either age group (proportion of work done by experienced ants: $z = -0.815$, $p = 0.415$; Table 3.4). However, for naïve ants, there is a statistically significant interaction between forager age and the proportion of work done by experienced ants (proportion of work done by experienced ants: $z = -2.888$, $p = 0.004$; Table 3.5).

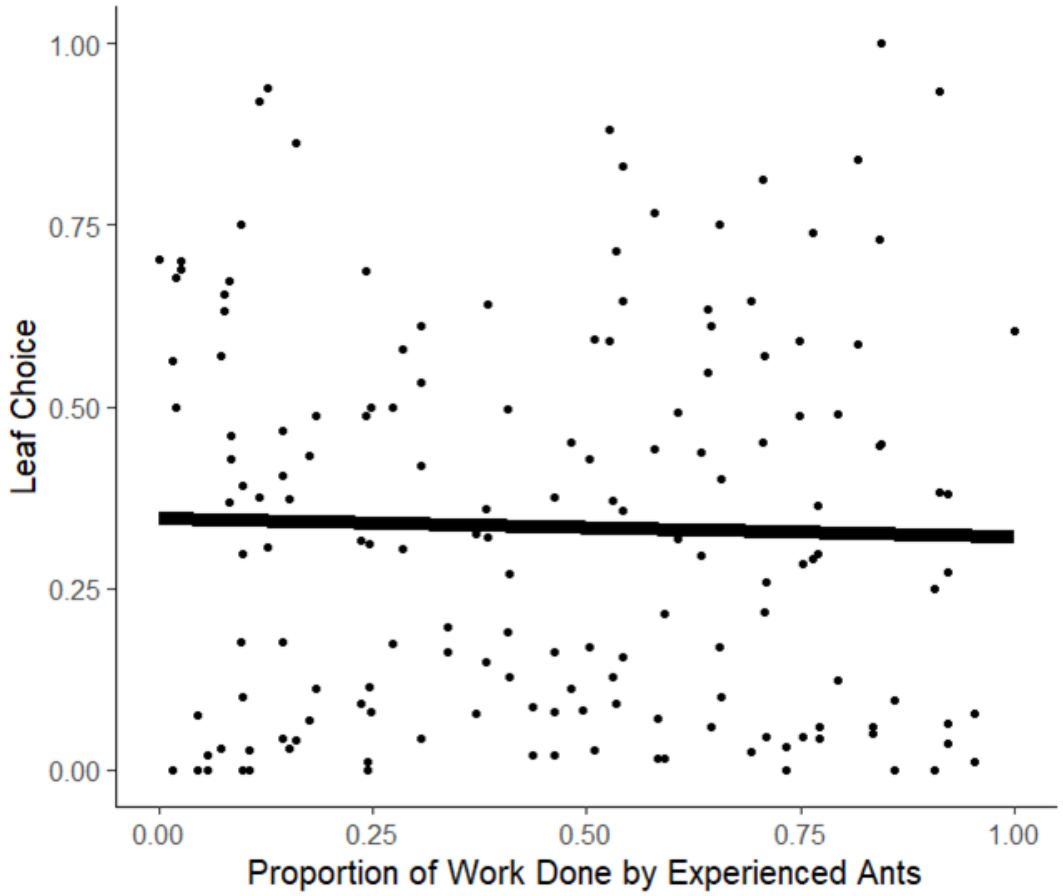

Figure 3.6 Without considering forager age and forager experience, there is no effect of the proportion of work done by experienced ants (Proportion of work done by experienced ants: $z = 0.650$, $p = 0.515$; Table 3.3). For each of the 40 trials there are two data points for old ants and two data points for young ants. Each age group has a data point for the first 4-hour time block and the second 4-hour time block. For any given trial age and experience are linked. The total sample size is 160.

Conclusions and Open Questions

I found that collective memory in leafcutter ant colonies is maintained due to overlapping age-cohorts within a colony (Chapter 2), a decrease in responsiveness to new social and asocial information by experienced foragers (Chapters 2 and 3), a decrease in conformity by older individuals (Chapter 3), and possibly an aversion to novelty in young, naïve individuals (Chapter 2). While maintaining 'inherited' caution, leafcutter ant colonies are also able to flexibly respond to changes in the environment due to worker turnover (Chapter 2), noisy copying (Chapters 2 and 3), and the interaction between personal and socially acquired information (Chapters 2 and 3).

In Chapter 1, I found two likely causes of mortality in *Atta texana* foundress queens; two types of entomopathogenic fungi. The mortality rate from the time of the mating flight to the emergence of the first workers was 83.7% in the laboratory. There was a weak correlation between worker number and fungus garden volume in the first few months of colony development, and a high degree of variation in both worker number and fungus garden volume.

In Chapter 2, I found that young, naïve foragers copy the leaf preferences of their old, experienced nestmates while these two groups forage together. This is likely accomplished via two positive feedback mechanisms: first, imitation of leaf choices detected on the foraging trail; and second, the presence (or lack of) leaves detected in the fungus garden. I tested, but did not find support for, three other possible negative feedback mechanisms: discouraging contacts received by foragers who are carrying 'bad' leaves, the presence of minims sitting on 'bad' leaves, and the removal of 'bad' leaves by experimenters with forceps. I use 'bad' in inverted commas here to mean that these leaves are believed to be harmful by experienced ants, but actually they were no longer harmful for the duration of the experiment. I also found that young, naïve ants re-accepted leaves of the previously treated type when they detected them (now harmless) in their gardens.

But, old, experienced ants were more stubborn (i.e., less likely to change their behavior) and maintained their leaf rejection when they had access to the same information.

In Chapter 3, I investigated the social learning strategies that ants use to accomplish the collective leaf-rejection patterns that I observed in Chapter 2. I found that both young and old ants that had experienced fungicide treatment were not responsive to any sources of social information. Old naïve ants decreased their collection of leaves of the previously treated type slightly when foraging with experienced minim ants, and slightly over the course of the day. However, old, naïve ants responded to, but did not conform to, the leaf preferences of young, experienced ants when young, experienced ants made the majority of leaf choices.

Demography and social information use

These studies highlight the importance and potential roles of overlapping generations in the maintenance and flexibility of collective memory. In my study and also in humans (Gopnik 2020), older individuals are less behaviorally flexible when presented with new information. As a result, older individuals may preserve accumulated knowledge. In my study and in other groups, younger individuals are more prone to copy others (Slagsvold and Wiebe 2011; Perry and Perry 2020). But, importantly, this copying is often noisy and imperfect (Sasaki and Biro 2017). Imperfect copying and higher responsiveness to changing environments allows for younger individuals to preserve the most useful knowledge from previous generations and to innovate at a moderate pace (Sasaki and Biro 2017; Aplin 2019).

At the individual level, leafcutter ants and other animals (Valone 2006) appear to be Bayesian in nature. In other words, the more information an animal has, the less likely it is to change its behavior when presented with new and conflicting information. When this behavioral

rule exists in the context of the constant, gradual turnover of individuals, the system is both flexible and capable of retaining information. In other words, age-based inflexibility in individuals' behavior creates tempered flexibility at the level of the colony or population.

Are there colonies that themselves accumulate knowledge over many age-cohorts of workers? In leafcutter ants, when a type of leaf becomes harmful, a colony may sample it initially at high levels, believing it to be beneficial. But if such leaf types remain harmful, the colony will reject harmful leaf types and thereafter sample at very low levels. This inherited caution could be maintained until such leaves were to become again beneficial for the fungus garden. In this way, leafcutter ant colonies may accumulate learned caution towards many types of leaves such that this caution persists across worker age-cohorts, until leaf quality changes.

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